

AWARD NUMBER: W81XWH-12-1-0506

TITLE: Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders

PRINCIPAL INVESTIGATOR: Georgianna G. Gould, M.S., Ph.D.

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13. SUPPLEMENTARY NOTES A modification of the contract to provide a 6 month no-cost extension until 31 March 2016 was granted on 14 Aug 2015. The final technical report is now due on 29 June 2016.					
14. ABSTRACT Two populations of membrane-bound transporters clear serotonin from brain extracellular fluid: "Uptake 1" are high-affinity sodium-dependent transport proteins which include the serotonin transporter (solute carrier 6 member 4), the target of Prozac. By contrast "Uptake 2" are low affinity, high capacity transporters that include organic cation transporters (solute carrier family 22, types 1,2 and 3). Our prior behavior findings in mice suggest targeting uptake 2 transporters holds promise as a strategy for treating sociability impairments in autism. The goal of this project is to characterize the behavioral, physiological and in vitro pharmacological effects of blocking uptake 2 transporters with decynium-22 in several strains of mice, including black and tan brachyury tufted (BTBR) mice with inherent sociability deficits and repetitive traits that parallel core autism symptoms. We dosed mice with 0.5 mg/kg D-22 and collected samples to measure its concentration in blood and brains at 15-min time points from 163 mice by GC/MS. The impact of D-22 on dominance and sociability preference alone and in presence of fluoxetine and risperidone continues to be assessed, but so far D-22 suppress dominance and enhances sociability. Measures of in vitro synaptosomal uptake of [ <sup>3</sup> H] serotonin and its blockade by D-22 and other compounds in these mice are nearly complete. A revised protocol for synaptosomal uptake of [ <sup>3</sup> H] histamine is being developed and [ <sup>3</sup> H] D-22 autoradiography is underway. Serotonin uptake blockade by D-22 seems to be the mechanism mediating the beneficial effects, but others can't be ruled out.					
15. SUBJECT TERMS autism, auxiliary monoamine transporters, corticosteroids, decynium-22, impulsivity, inbred mice, organic cation transporters, repetitive behavior, serotonin, social behavior, repetitive behavior					
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## INTRODUCTION:

The main goal of this project is to characterize the role of 'uptake 2' transporters in shaping social behavior. Uptake 2 transporters are a population of ancillary solute carrier proteins in the brain with substrates that include monoamine neurotransmitters such as serotonin (5-HT). We hypothesized blockade of uptake 2 transporters, including organic cation and plasma membrane monoamine transporters (OCTs and PMAT) would enhance sociability by inhibiting clearance of extracellular serotonin to a similar or greater extent than blockade of the serotonin transporter (SERT). Selective 5-HT reuptake inhibitors (SSRIs) are often used to treat the behavioral symptoms of autism; their function is to inhibit 5-HT uptake by blocking SERT to promote 5-HT neurotransmission in brain regions regulating social behavior.

Publications from our laboratory group demonstrate that monoamine clearance by uptake 2 transporters can dampen therapeutic benefits of SSRIs in depression-related mouse behaviors (Horton et al., 2013; Daws et al., 2013). This indicates that 'uptake 2' activity could likewise contribute to the poor efficacy of SSRIs to ameliorate social behaviors of autism. The pseudoisocyanine decynium-22 (D-22) is an effective blocker of uptake 2, so for **task 2 (aim 1)** we use it as a tool to examine effects on social preferences and serotonin uptake in four mouse strains differing in social behavior and SERT function: BTBR T+Itpr3 tf/J (BTBR) and 129S1/SvImJ (129S), with impaired sociability and high SERT function, versus gregarious C57BL/6J (C57) and DBA1 mice with either impaired or high SERT function. For task 2 (aim 1) we compared pharmacokinetics of D-22 (0.5 mg/kg) in brains from these mice to determine if D-22 reached sufficient concentrations to block uptake 2 between 50 and 100 min post-injection, when behavioral effects were evident. For **task 2 (aim 2)** in response to post-award suggestions from CDMRP reviewers, we continue to look for other compounds currently in clinical use that also block uptake 2 to confirm behavioral effects of D-22 are common to OCT blockade. We found that berberine, an antimicrobial compound with anti-diabetic and anti-cancer properties in use worldwide, is a substrate and blocker of organic cation transporters (Sun et al., 2014, Derosa & Maffioli 2014). Thus we compared its efficacy to enhance sociability versus D-22 in BTBR and C57BL/6 mice, and found it shared similar sociability enhancing properties with D-22. Also since fluoxetine improved social behavior in BTBR mice, we examined the efficacy of other selective serotonin reuptake inhibitors such as citalopram or vortioxetine to enhance sociability.

For **task 3 (aim 2)** we continued working to characterize efficacy of D-22 and berberine to block uptake of 5-HT in vitro. For example, we've achieved a sample size of 5 for determination of the Michaelis constant (Km) for D-22 to block [<sup>3</sup>H] 5-HT uptake in synaptosomes, that is  $632 \pm 168$  nM BTBR mice as compared to  $56 \pm 14$  nM for fluoxetine. The antimicrobial herbal compound berberine was reported to be a substrate and blocker of OCT2 & OCT3, albeit with lower affinity than D-22 (Sun et al., 2014; Derosa et al., 2014). We found the Km for berberine to block [<sup>3</sup>H] 5-HT uptake in vitro was  $80 \pm 15$  uM, so it was a less potent blocker of [<sup>3</sup>H] 5-HT uptake than D-22. Protocols to measure blockade of [<sup>3</sup>H] histamine uptake by D-22 are being developed in our lab, since it is a high-affinity OCT substrate and could be a useful tool for teasing apart the contributions of different uptake 2 transporters. Metformin was just identified as a substrate of OCT 3 (Lee et al., 2014). Therefore we requested an extension of research time on this project to examine metformin's ability to measure OCT function.

For **task 4 (aim 3)** we continued to examine the effects of D-22, risperidone, fluoxetine and their combinations on social dominance by means of the tube test. We have been using marble burying and grooming during three chamber sociability tests as indices of restrictive-repetitive behaviors in three of the mouse strains in this study, and have examined the capacity of D-22 vs. risperidone or fluoxetine to reduce them, but none were as effective as risperidone.

**KEYWORDS:** autism, monoamines, dominance, serotonin, social behavior, transporters

## **ACCOMPLISHMENTS:**

### **Task 1: Regulatory Approvals**

An Institutional Animal Care and Use (IACUC) progress report was submitted for review on August 7, 2015 and was approved on September 4, 2015. Use of mice for this project is therefore approved to continue through August 21, 2016. We hope to continue this line of research in case another line of funding is secured for it, possibly through a training program.

Relevant IACUC correspondence and current letter of approval can be found in Appendix A.

Additionally an amendment was requested and granted to extend the deadline for this project at no additional cost through March 31, 2016 so that ongoing experiments can be completed and manuscripts written. The contract amendment is included in Appendix B.

### **Task 2: Pharmacokinetic & Behavioral Characterization of D-22 in Mice**

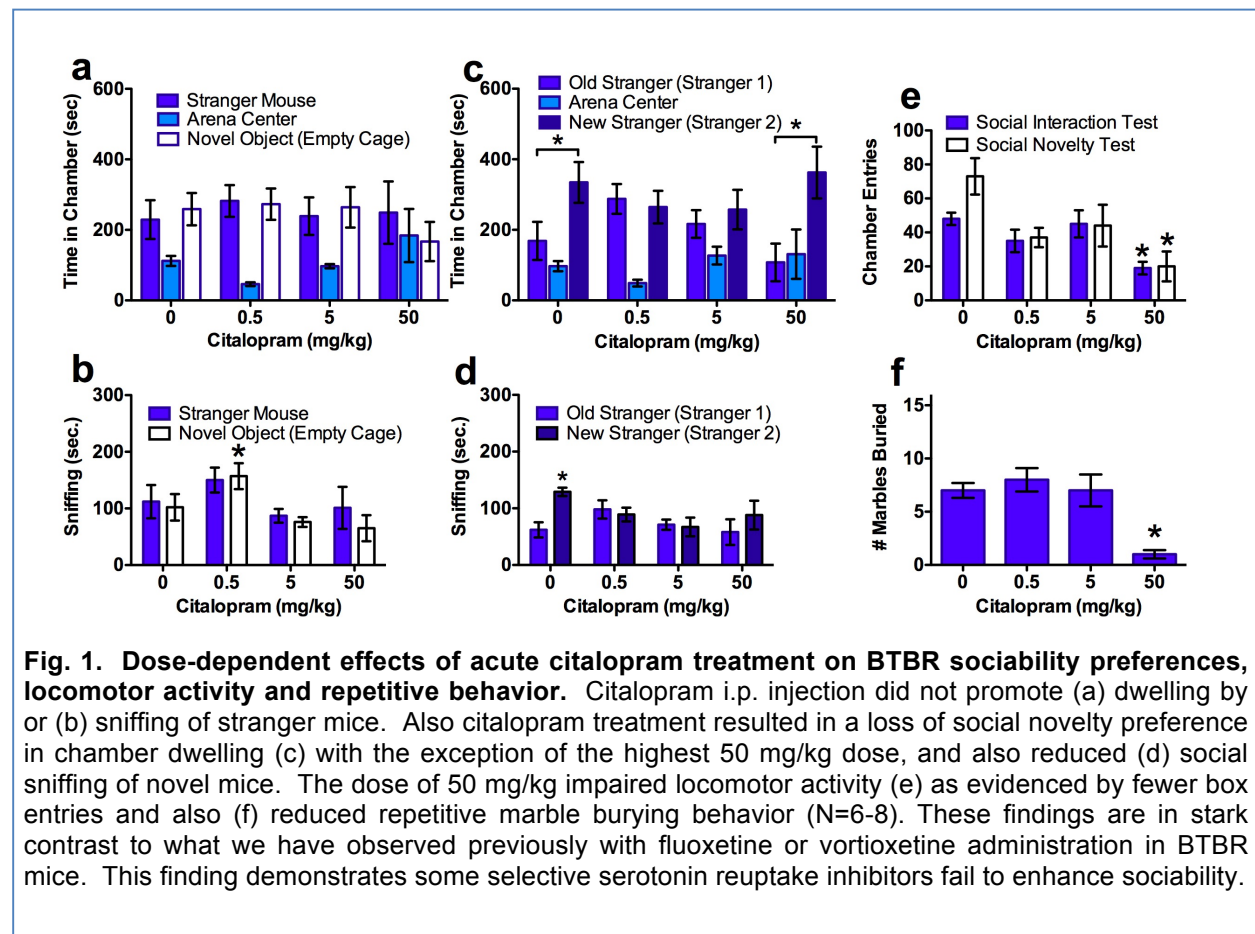
#### Aim 1a: Pharmacokinetics of D-22 in mouse brain and blood:

This year, measurements of intraperitoneally-injected D-22 at a dose of 0.5 mg/kg was performed in 163 mice of strains BTBR, C57BL/6, 129S1/SvImJ and DBA1. The mice were sacrificed at 1, 15, 30, 45, 60, 75 and 100 min after injection. Whole brains were collected for measurement by GC/MS and serum by HPLC. The GC/MS approach was under development for most of the year, and now appears to be rendering accurate measures. So far roughly half of the brain tissue has been analyzed, revealing brain D-22 concentrations of  $5 \pm 1$  pm/mg in C57BL/6 (N = 25),  $2.5 \pm 1$  pm/mg tissue in DBA (N = 6),  $6 \pm 1$  pm/mg tissue in 129S (N = 33), and  $5 \pm 2$  pm/mg tissue in BTBR mice (N = 22). Since there is little variability across time-points in brain D-22 concentrations, it appears these injections reach sufficient concentrations in the brain to occupy either the target organic cation transporter (OCT), plasma membrane monoamine transporter (PMAT) binding sites within a minute of injection, and these concentrations are sustained throughout the timeframe of behavior testing. This finding confirms our prior published account that D-22 injected systemically at 10 mg/kg enters the brain (Horton et al., 2013). D-22 in the remaining brain tissue samples will be measured by GC/MS, and in the serum samples will be measured by HPLC between now and March 31, 2016.

#### Aim 1 b & 3. Dose-response in three-chamber sociability tests for D-22 and drug combinations:

Acute treatment of BTBR, C57BL/6, 129SvImJ or DBA1 mice continued using D-22 in comparison to fluoxetine, risperidone, berberine or vehicle control treatments in three chamber social interaction and novelty preference tests. The dose of 0.001 mg/kg was examined in greater detail, since prior results had large standard errors due to low sample size. Most of the behavior was performed and videorecorded several months ago, however data is still being collected from these videos. The goal for these data is to complete the very large data set collected over the past three years in preparation for a manuscript submission in Spring 2016.

We examined the ability of selective serotonin reuptake inhibitors other than fluoxetine to enhance sociability in BTBR mice. We found that acute citalopram administration at a range of doses failed to enhance social behavior (Fig. 1) while vortioxetine was able to transiently enhance sociability, as we reported in a manuscript currently under review (Appendix C).



Additionally, in researching other treatments in common medical use that are blockers of OCTs, we came across berberine as an alternative for D-22. We have already established that in three-chamber sociability tests the uptake 2 blocker decynium-22 (D-22) improves social behavior in otherwise socially-impaired BTBR T+/tf mice. The alkaloid antibiotic berberine also has antidepressant-like properties in mice, and is both a blocker and substrate of OCTs. Given this, we hypothesized berberine might improve the sociability of BTBR mice as D-22 does. Indeed, berberine significantly increased BTBR preference for social interactions and social sniffing ( $p < 0.05$ ,  $N=8-9$ ). Furthermore it enhanced the already sociable behavior of C57BL/6 mice, but it had no impact on repetitive marble burying in either strain. Our findings confirm that systemic OCT3 and uptake 2 blockade is a promising strategy for improving social behavior warranting further investigation as a treatment for sociability impairments. These data are summarized in a poster presented in Appendix D.

In that same Appendix D poster, we present data comparing the social behavior preferences of naïve C57BL/6 male mice from different suppliers (Harlan vs. Jackson), and found subtle differences in novelty preference. This data was prepared as a manuscript last year that was not accepted for publication. Reviewer concerns were that its sample size was insufficient. The sample size was doubled this year, and results were similar. Plans are to either resubmit the

data to that journal, or to include it in a D-22 manuscript involving serotonin transporter knock-out mice that is under preparation for submission to a journal with greater impact.

#### Aim 1 c. Measures of serum corticosterone levels following behavior tests by EIA

We have collected a large number of serum samples from drug treated mice, and plan to acquire more ELISA kits to measure the corticosterone levels in these by February 2015.

### Task 3: Serotonin Uptake Studies *in vitro* and *in vivo* in mice

#### Aim 2a. Effects of D-22 on [<sup>3</sup>H] serotonin uptake *in vitro*

We have continued to make good progress toward characterizing D-22's ability to block serotonin (5-HT) uptake in each of the four mouse strains, in both the frontal cortex and hippocampus, as shown in **Table 1**. Three more experiments are planned using 129S, DBA and C57BL6 mice to complete the data set for publication. Risperidone by itself has no affinity to block [<sup>3</sup>H] 5-HT uptake.

Table 1. Affinities of D-22 and other compounds to block serotonin (5-HT) uptake. Michaelis constant values (Km) reported in nM units. The [<sup>3</sup>H] 5-HT concentration was 25 ± 1.2 nM.

#### Hippocampus

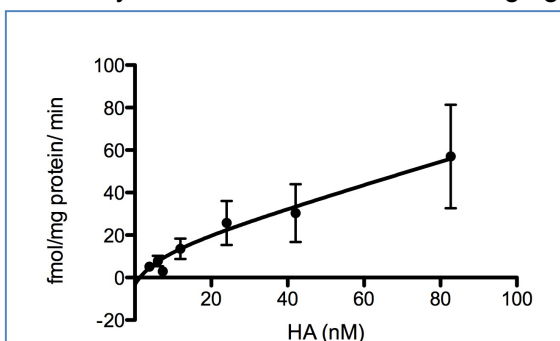
Mouse strain	Fluoxetine	D-22	D-22 + fluoxetine	D-22 + risperidone
BTBR	56 ± 14	632 ± 168	135 ± 79	865 ± 195
129S1/SvImJ	88 ± 22	486 ± 143	34 ± 14	846 ± 464
C57BL/6J	289 ± 160	953 ± 227	76 ± 15	905 ± 333
DBA1	165 ± 113	721 ± 198	354 ± 316	785 ± 45

#### Frontal Cortex

Mouse strain	Fluoxetine	D-22	D-22 + fluoxetine	D-22 + risperidone
BTBR	90 ± 36	778 ± 117	71 ± 7	826 ± 56
129S1/SvImJ	40 ± 10	458 ± 128	53 ± 12	648 ± 186
C57BL/6J	234 ± 183	664 ± 3	56 ± 2	567 ± 104
DBA1	41 ± 18	444 ± 14	100 ± 29	678 ± 124

#### Aim 2b. [<sup>3</sup>H] Histamine uptake and D-22's effects

This assay has continued to be challenging to develop, in C57BL6 mice we obtained saturable binding, but the Vmax value was low (16 fmol/mg



**Fig. 2. Histamine uptake in cerebellar synaptosomes.** Binding was saturable yet the Vmax for uptake is low relative to 5-HT.

protein/min) in cerebellar synaptosomes, with a Km of 7 nM (Fig 2). This raises some concern about the utility of this assay to be used for competition assessments. We have yet to utilize SKF 91488 dihydrochloride (Tocris, Bristol, UK) in our assays to reduce enzymatic breakdown of HA. We also continue to work with gradients and centrifugation to separate synaptosomes from astrocytes. However, a new publication demonstrates that metformin, the antidiabetic drug, is a high affinity substrate of organic cation transporters (Lee et al., 2014). Given this, and in light of an ongoing clinical study examining use of

metformin to control antipsychotic induced weight gain (IMPACT study, Reeves et al., 2013), we will examine *in vitro* its uptake properties in synaptosomes and blockade by D-22 this year.

#### Aim 2 c. In vivo chronoamperometry to measure the effect of D-22 on 5-HT uptake

Three more mice were analyzed for this aspect of the project this year. The data collected did not substantially change the findings from last year's report.

#### **Task 4: Measuring D-22 impact on other behaviors and characterizing its binding sites**

For aim 3 we continue to characterize behavioral effects of D-22 in the tube test for social dominance wherein 129S1/SvImJ mice are used as stimulus 'competitors'. Generally D-22 reduces the number of wins the treated mice had relative to vehicle controls and shortened the duration of those matches, as do selective serotonin reuptake inhibitors. D-22 reduced self-grooming during 3 chamber sociability tests, but does not reduce marble burying by BTBR mice.

#### **Stated Goals Not Met**

Western blotting for uptake 2 and serotonin transporters in the brain was not possible since no reliable antibodies are currently available for many of them to examine in the mouse brain. Also we have not been able to progress with [<sup>3</sup>H] histamine uptake, but will explore use of alternative substrates like [<sup>3</sup>H] MPP+ or [<sup>3</sup>H] metformin. It may be possible to use metformin to define non-specific binding by [3H] D-22, this will be determined in the next few months.

#### **Opportunities for Training and Professional Development**

Georgianna Gould, PI, was promoted on 9/1/2015 to associate professor, research track. Also Dr. Gould was interviewed by the University of Texas at San Antonio for a tenure-track assistant professor position of neuroscience, but was not selected for the position. Finally Dr. Gould was again invited to review abstracts for the INFAR autism meeting and to serve as an ad-hoc member of a committee reviewing grants for the National Science Foundation.

Corey Smolik continues to work at the University Hospital as an emergency room technician.

Wynne Q. Zhang, now a junior at Rice U. served as a student researcher in the lab. She has scored well on her MCAT exam and was a first author on a manuscript related to this research.

Sergey Poplyaev was hired as a Research Laboratory Technician to replace Corey Smolik in March 2015. He managed mouse colonies and learned to perform three chamber tests, but resigned from this position on June 23, 2015 due to the demands of the job being too great.

Marshall Edwards was hired on May 4, 2015 as a research assistant to replace Sergey Poplyaev and quickly took over mouse colony management, behavior tests, video analysis and is now learning to perform uptake and binding assays in vitro. He was accepted into a master's program in immunology and infection at UTHSCSA and now has dual appointment as a student and research assistant.

Clover Moten a high school student from San Antonio who worked on this project as part of her Voelcker Foundation Scholarship, she will graduate this year and will attend college in the fall.

Alicia Sanchez graduated from St. Mary's University and is taking a year off to perform missionary service for her church. As a student researcher in the lab, she performed work on this project and made poster presentations to disseminate results at local meetings. Next year



she will apply for graduate school, and her experience through this program and authorship on manuscripts should strengthen her candidacy.

Benita Lee is a high school student from San Antonio who worked on this project as part of her Voelcker Foundation Scholarship, she is the primary author on the Vortioxetine manuscript, and will return this summer to continue working on manuscripts from this project.

## **Dissemination of Results**

### Invited Research Oral Presentations:

12/2015 Dept. Biology, William Paterson University, Wayne NJ, "Next Generation Uptake Inhibitors: Targeting Ancillary Monoamine Transporters"

10/2015 Chicago IL, Society for Neuroscience, #467, Hormones, Neurotransmitters & Social Behavior, "Uptake 2 Transporter Blockade Ameliorates Deficits in Sociability in Two Mouse Models"

10/2015 Dept. of Physiology, UT Health Science Center, San Antonio, TX, "Sustained Uptake 2 Blockade and Sociability in an Insulin-Resistant Mouse Model of Autism"

7/2015 Voelcker Scholars Research Presentation, UT Health Science Center, San Antonio, TX "Serotonin's Roles in Shaping Social Behaviors"

3/2015 UTSA Biology, San Antonio, TX "Serotonin as a key shaper of social behavior"

### Posters at Meetings:

Gould, GG 2015. "Uptake 2 Transporter Blockade Can Ameliorate Sociability Deficits", International Meeting for Autism Research, Salt Lake City, UT, May 15, 2015 Poster # 139.149, Session: Interventions Pharmacologic.

Gould, GG 2014. "Decynium-22 Enhances Social Behavior in Serotonin Transporter Knock-Out Mice. American College of Neuropsychopharmacology, 53<sup>rd</sup> Annual Meeting, Phoenix, AZ, December 10, 2014. Poster # W188.

Sanchez A, Smolik C, Pham T, Lalani K, G. Gould. Berberine blocks 'uptake 2' and enhances mouse sociability. UTHSCSA Mikitin Research Forum, San Antonio, TX, May 11, 2014.

### Manuscripts under review:

Lee B, Pehrson A, Witt N, Allen J, Sanchez C, Gould GG. Vortioxetine inhibits marble burying and transiently enhances social sniffing in adult male BTBR mice. Autism Research, 15-Dec-2015.

### Plan for Final Reporting Period:

1. Complete all behavior studies with acute drug treatments in all mouse strains.
2. Complete [<sup>3</sup>H] serotonin uptake studies in vitro and explore use of metformin as a substrate that can be blocked by D-22.
3. Perform corticosterone enzyme immunoassays (EIAs).
4. Submit two new manuscripts for peer review and publication in journals based on studies described in this and previous progress reports.

**IMPACT:**

The findings from this project and its publications highlight the importance of serotonin neurotransmission in the brain in shaping adult social behavior, and how it is sensitive not only to selective serotonin reuptake inhibitors, but also to blockade of ancillary transporters of serotonin. Among these organic cation transporters (OCTs) and plasma membrane monoamine transporters (PMAT) are blocked by D-22 that reached the brain following acute systemic administration and likely mediates its behavioral effects. These findings might also be extended to other fields within biomedical neuroscience such as schizophrenia or depression wherein impaired social behavior is prominent.

**Technology Transfer:**

Nothing to report.

**Commercial Technology**

Nothing to report.

**CHANGES/PROBLEMS:**

Western blot could not be performed for OCTs in mouse brain due to poor performance of commercially available antibodies. [<sup>3</sup>H] histamine uptake into synaptosomal preparations was not robust enough to be used to tease apart contributions of OCTs from PMAT. We will perform assays using metformin instead to assess its use as a selective substrate of OCTs.

***Actual or anticipated problems or delays and actions or plans to resolve them***

Due to the difficulties with [<sup>3</sup>H] histamine assays, and due to challenges associated with research assistant turnover and project understaffing from August 2014 – May 2015, a no cost extension was requested through March 31, 2016 to complete the project and prepare manuscripts.

Deviations in reporting and IACUC:

On January 26, 2015 the University of Texas Health Science Center (UTHSCSA) Institutional Review Board (IRB) provided us with notice of an Institutional Animal Care and Use Committee (IACUC) report of non-compliance of the rodent housing policy due to overcrowding of mouse breeding cages that they received from Laboratory Animal Research (LAR) staff on January 8, 2015. In response to this, the PI complied with the stipulations by: 1) providing written documentation for why the cage overcrowding occurred, 2) provided a new standard operating procedure for colony management, and 3) completing additional colony management training with veterinary staff in January 2015.

A substantial increase in mouse breeding on the protocol was planned and initiated in October - November 2014 so sufficient numbers of adult male mice from each strain would be available to accommodate the last phase of pharmacokinetic measures in Summer 2015. As many of the mouse litters were being born, the PI missed several days of work due to an ongoing family medical issue, and during this time males were not removed from breeding cages in time to prevent second litters. The overcrowding events occurred during November - December 2014, and it was resolved by the first week of January, 2015. The animal colony was observed daily for overcrowding events by LAR staff and veterinarians until a new employee was hired and they were satisfied with our updated practices in colony management.

The IACUC non-compliance report necessitated additional administrative actions to maintain approval for ongoing studies with protocol 12069x. The UTHSCSA IRB notified ACURO of the

non-compliance report on February 11, 2015. On May 28, 2015 IACUC provided to the PI an "Approval of Response" to IACUC stipulations. These are included in Appendix A.

Significant changes in use of biohazards and/or select agents  
Nothing to Report

## **PRODUCTS:**

Manuscripts requiring revision and resubmission (See Appendix C):

Lee B, Pehrson A, Witt N, Allen J, Sanchez C, Gould GG. Vortioxetine inhibits marble burying and transiently enhances social sniffing in adult male BTBR mice. Autism Research, Submitted 15-Dec-2015.

Books or other non-periodicals:  
Nothing to Report.

Technologies or techniques:  
Nothing to Report

Other Products:

In addition to other oral presentations and posters reported in the "Dissemination of Results" the PI will chair a symposium at the "Serotonin in Seattle" meeting in July 2016. The title of the symposium will be "The highs and lows of serotonin in autism spectrum disorders". The meeting is hosted by the International Society of Serotonin Research. (Appendix E).

Poster Presentation: Gould G "Influence of Central Serotonin Availability on Social and Repetitive Behavior in Mice" Abstract #1319 Experimental Biology 2016 Meeting San Diego.

## **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:**

1. Project Director/Principal Investigator

Name: **Georgianna Gould**

Project Role: Principal Investigator

Researcher Identifier: gouldg (ERA Commons)

Nearest person month worked: 4.8 mos/yr, will change to 1.2 mos/yr for extension

Contribution to Project: Dr. Gould is the PI on the project and is responsible for performing or overseeing the performance of all aspects of the project.

W81XWH-12-1-0506 (Gould, PI)

09/30/12 – 03/31/2016

Autism Idea Award

AR11019 CDMRP/DOD

\$125,000 Annual Direct

*Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders*

The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Gould, Other Support:

Role: PI, 0.12 mos/yr

Lindow, Stevens & Treat Research Award, 2/1/2012-7/30/2016, \$40,000/yr

Title: Neuroprotection from pesticide-induced sensitization via transporter blockade

Goals of project: To examine in zebrafish if up-regulation of uptake 2 transporters contributes to pesticide-induced sensitization to neurotoxins, and if their blockade is neuro-protective.

Role: PI, 2.4 mos/yr

NIH R21HD081261-01 (Gould), 4/1/2015-3/31/2017, \$125,000-150,000/yr

Title: Impact of Gestational Serotonin Availability on Brain Function & Social Behavior

Goals of project: To study the impact of limiting gestational serotonin availability on brain development and social behavior.

Role: Collaborator, 1.9 mos/yr

5R01MH093320-02 (Co-PIs Daws/Koek), 3/1/2012 – 11/30/2016, \$350,000/yr

Title: *Organic cation transporters as targets for novel antidepressant drugs*

Goals of project: To examine the efficacy of OCT3 blockade as an antidepressant *in vitro* and *in vivo*.

Role: Collaborator, 3.24 mos/yr

1R01MH106978-01 (PI Daws), 5/1/2015 – 11/30/2016, \$417,471/yr

Title: *Age-related differences in serotonin clearance: Novel targets for antidepressants*

Goals of project: To investigate expression and activity of transporters for serotonin, including the high-affinity serotonin transporter (SERT), as well as the low-affinity, high-capacity organic cation transporters (OCTs) and plasma membrane monoamine transporter (PMAT), in juvenile and adolescent mice.

## 2. Other Effort-Contributing Researchers

Name: **Marshall Edwards**

Project Role: Research Assistant

Nearest person month worked: 6 mos/yr

Contribution to Project: Assisted Dr. Gould by maintaining mouse colonies, performing radioligand uptake and binding assays, performing ELISA assays for serum corticosterone, and collecting data from behavior videos.

Other Support: Dr. Gould's LST Research Award

Nearest person month worked: 6 mos/yr

Contribution to Project: Performed radioligand binding assays, repaired aquatic habitat

Name: **Alicia Sanchez**

Project Role: Undergraduate Research Assistant

Nearest person month worked: 1 mos/yr

Contribution to Project: Assisted Dr. Gould by measuring autoradiograms, performing behavior tests and collecting data from behavior videos.

Other Support: None

Name: **William Anthony Owens**

Project Role: Senior Research Associate

Nearest person month worked: 0.12 mos/yr

Contributions to Project: Performed *in vivo* chronoamperometry in BTBR mice.

Other Support: 5R01MH093320-02 (Co-PIs Daws/Koek)

Contribution to Project: *In vivo* chronoamperometric recordings.

Name: **Lynette C. Daws**

Project Role: Co-Investigator

Researcher Identifier: daws (ERA Commons)

Nearest person month worked: 1.2 mos/yr

Contribution to Project: Dr. Daws oversees performance of chronoamperometry and makes intellectual contributions to uptake assays she also makes her laboratory space and personnel available for these studies.

W81XWH-12-1-0506 (Gould, PD/PI)

09/30/12 – 09/29/2015

Autism Idea Award AR11019 CDMRP/DOD

\$125,000 Annual Direct

Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders

The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Daws Other Support:

**1R01 MH106978-01** (Daws)

05/2015 – 03/2019 3.6 calendar months

NIH/NIMH

\$417,471

*Age-related differences in serotonin clearance: Novel targets for antidepressants.*

These studies investigate the expression and activity of transporters for serotonin, including the high-affinity serotonin transporter (SERT), as well as the low-affinity, high-capacity organic cation transporters (OCTs) and plasma membrane monoamine transporter (PMAT), in juvenile and adolescent mice. These studies take advantage of mice with constitutive reductions or knockout of SERT, OCT3 or PMAT to understand mechanisms contributing to serotonin clearance in brain during these young ages, with the goal to understand the mechanistic basis for lack of therapeutic efficacy of SSRIs in juvenile and adolescents who suffer depression, and to identify new targets for the development of antidepressants with improved therapeutic efficacy for this young population.

*Role: Principal Investigator*

1R01 MH093320-01 (Daws and Koek)  
months

03/2012 – 11/2016 3.6 calendar

**NIH/NIMH**

\$357,363

*Organic cation transporters as targets for novel antidepressant drugs*

Experiments in this study are designed to validate organic cation transporter-3 (OCT3) as a target for the discovery of drugs with improved therapeutic potential for the treatment of depression in adults. In these studies we test analogs of decynium-22, the prototypical blocker of OCTs. These analogs vary in their affinity for OCT3 and will be used, together with OCT3 KO mice, to investigate the contribution of OCT3 to serotonin, norepinephrine and dopamine uptake in adult brain, and the consequence of their blockade for antidepressant-like behavior.

*Role: Co-Principal Investigator*

1R21 DA038504 (Daws)  
months

06/2014 – 05/2016 2.4 calendar

**NIH/NIDA**

\$123,125

*The dopamine transporter in eating disorders: Uncovering novel therapeutic targets.*

Eating disorders, including anorexia, bulimia nervosa and binge eating, are major public health concerns, particularly prevalent in adolescent girls, and are compounded by a lack of effective treatments. Dysfunction of dopaminergic neurotransmission is implicated in these illnesses, but

studies investigating the relationship between the dopamine transporter, a primary regulator of dopamine neurotransmission, and eating disorders during adolescence and adulthood are lacking. These studies will begin to fill critical knowledge gaps, with the long-term goal to elucidate novel targets for the treatment of these debilitating and often fatal disorders.

*Role: Principal Investigator*

R13 DA033783 (Daws) 04/2012 – 03/2017 0.12 calendar months  
**NIH/NIDA** \$75,000

*Serotonin Club Meetings 2012-2016*

This award provides funds to offset travel expenses for young investigators (students, post-docs and junior faculty not more than three years past their post-doc) to attend the 2012, 2014 and 2016 meetings of the International Society for Serotonin Research (ISSR), formerly, the Serotonin Club. There is no salary commitment.

R01 DA026947 (Khoshbouei) 07/2015 – 04/2017 0.36 calendar months  
**NIH/NIDA** \$15,239 (subcontract component)

*Methamphetamine regulates the dopamine transporter via an intracellular mechanism*

These studies investigate sigma receptor regulation of the dopamine transporter using in vivo electrochemical approaches.

*Role: Principal investigator of subcontract*

**Center for Biomedical Neuroscience Pilot Grant (Paukert)**

01/2015 – 12/2015 0.12 calendar months

University of Texas Health Science Center \$50,000

*Electrochemical tracking of behavioral state-dependent neuromodulation.*

This pilot study aims to develop fast-scan cyclic voltammetry to measure norepinephrine release in primary visual cortex in head-fixed, awake mice following ambulatory and visual stimulation.

*Role: Co-Investigator*

Name: **Martin A. Javors**

Project Role: Collaborator

Researcher Identifier: javors (ERA Commons)

Nearest person month worked: 1.2 mos/yr

Contribution to Project: Dr. Javors measures tissue levels of decynium-22 and other drugs for pharmacokinetic studies, and also measures brain levels of monoamines by HPLC.

W81XWH-12-1-0506 (Gould, PD/PI) 09/30/12 – 09/29/2015  
 Autism Idea Award AR11019 CDMRP/DOD \$125,000 Annual Direct

Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders

The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Javors Other Active Support:

**R01-AA022361 (co-PIs: Javors/ Dougherty)** 9/2013 - 6/2017

NIAAA \$200,000 *Role: Co-Principal Investigator (20%) – 2.4 months*

Title: Phosphatidylethanol and Other Ethanol Consumption Markers

This study is designed to characterize and validate phosphatidylethanol as a biomarker for alcohol consumption and then determine how it alone (and in combination with 3 other alcohol biomarkers) can be used to identify an individual's level and pattern of drinking.

**P30AG013319-20 (PI: Javors)**

6/1/2014 – 6/30/2015

NIA \$65,000

*Role: Principal Investigator (3%)- 0.36 months*

Title: Bioanalytical Pharmacology Core, Nathan Shock Center, Barshop Institute

The purpose of the center is to provide analytical support to research groups for aging studies. Measurement of drug levels in dosage forms (usually food) and animal blood and tissues is provided in addition to assistance with study design is offered.

**5UO1-AG022307-09 Strong (PI)**

09/01/2014 - 08/31/19

NIA \$1M

*Role: Co-Investigator (30%)- 3.6 months*

Center for Testing Potential Anti-aging Interventions

The purpose of the center is to participate in a cooperative study to test interventions for which therapeutic targets have been identified that have been shown to control the aging process.

**3R01-AA014988-11S2/NIH Dougherty (PI)**

05/10/14 - 03/31/15

NIMH \$400,000

*Role: Co-Investigator (8%)-0.96 months*

Impulsivity and Biological Markers for Suicidality and Drug Use in Adolescents

This 5-year longitudinal study is designed to examine the interrelationships among impulsivity, 5-HT, stressful life events and the outcomes of drug use and suicidality in high-risk adolescents.

**2R01-MH076929-06A1 Xin-Yun Lu (PI)**

04/01/06 - 07/31/17

NIMH \$225,000

*Role: Co-Investigator (5%)-0.6 months*

Characterization of leptin's antidepressant activity

The goals of this renewal project are to determine the key components of glutamate neurotransmission that are responsive to leptin signaling and are responsible for the antidepressive effects of leptin.

**1R21-MH097092-01 Bowden (PI)**

04/25/12 - 03/31/16 (no cost extension)

NIMH \$125,000

*Role: Co-Investigator (2%)-0.24 months*

Calcium Study of Lymphoblasts in Bipolar Patients to Aid Diagnosis and Treatment

The overall goals of this project are to study the regulation of calcium activity in immortalized lymphocytes (LCLs) to develop a biological component as part of the diagnoses of bipolar disorder, resulting in more personalized, effective treatments and outcomes of bipolar disorder.

**W81XWH-08-2-0117 PTSD MRC Roache (PI)**

09/01/08 - 08/31/15

DOD-CDMRP- STAR PTSD Res Cnsrtium \$319,180 *Role: Co-Investigator (8%)-0.96 mos*

SSRI Treatment of Alcohol PTSD Dual Diagnosis – A Test of the Serotonin Hypothesis

This is an outpatient treatment trial evaluating the clinical utility of alcoholism subtyping to predict response to the use of sertraline to treat OEF/OIF veterans with dual diagnosis.

Name: **Wouter Koek**

Project Role: Collaborator

Researcher Identifier: koek (ERA Commons)

Nearest person month worked: 0.6 mos/yr

Contribution to Project: Dr. Koek provides space for animal behavior studies and statistical consultation and other intellectual contributions as needed.

W81XWH-12-1-0506 (Gould, PI)

09/30/12 – 09/29/2015

Autism Idea Award                      AR11019 CDMRP/DOD                      \$125,000 Annual Direct  
*Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders*  
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Koek Other Active Support:  
1R01 MH093320 (Daws, Koek)                      03/01/12 – 11/30/16  
NIH/NIMH                      \$1,785,000 - 3.6 cal mo  
Organic Cation Transporters as Targets for Novel Antidepressant Drugs  
The results of the studies under this grant will help to establish OCT3 (and/or PMAT) as a novel target for the discovery of drugs with improved therapeutic potential, as well as provide a mechanism that can, at least in part, account for poor therapeutic response to current antidepressant drugs.

R01 DA05018 (France)                      03/15 – 03/20  
NIH/NIDA                      \$225,000 – 0.6 cal mo  
Discriminative Stimulus Effects of Opioid Withdrawal  
This grant examines interactions between morphine and serotonergic (e.g., fluoxetine) or cannabinoid (e.g., THC) drugs to determine whether the combination enhances their ability to alleviate pain without increasing, and possibly decreasing, their abuse and dependence.

R01 MH106978-01 (Daws)                      05/15 – 05/20  
NIH/NIMH                      \$417,000 – 2.4 cal mo  
Age-related differences in serotonin clearance: novel targets for antidepressants  
Depression is a major public health problem, especially in young people, because the few antidepressants that are available to treat children and adolescents are less effective than in adults. These antidepressants block uptake of serotonin in brain by the serotonin transporter. The goal of this project is to investigate the role of other, “non-traditional” transporters for serotonin in limiting the therapeutic effects of antidepressants, especially in children and adolescents, with the goal to guide development of more effective antidepressants to treat young people.

R01 DA029254 (France)                      03/16 – 03/21  
NIH/NIDA                      \$225,000 – 1.2 cal mo  
Delay discounting: effects of drug dependence and withdrawal  
The goal of this project is to examine the effects of chronic drug administration and its discontinuation (withdrawal) on delay discounting to determine how common drugs of abuse affect impulsivity.

Name: **Julie Hensler**  
Project Role: Collaborator  
Researcher Identifier: hensler (ERA Commons)  
Nearest person month worked: 0.6 mos/yr  
Contribution to Project: Dr. Hensler provides critical equipment for uptake studies, consultation for planning experiments and manuscripts, and other intellectual contributions as needed.

W81XWH-12-1-0506 (Gould, PI)                      09/30/12 – 09/29/2015



Autism Idea Award                      AR11019 CDMRP/DOD                      \$125,000 Annual Direct  
*Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders*  
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Hensler Other Active Support:

R01 NS065783 (Mazarati, PI)                      05/01/2014-04/30/2018  
Role: Co-Investigator 0.6 calendar  
NIH sub through UCLA                      \$10,000 Annual Direct Costs

*Mechanisms of Co-Morbidity between Epilepsy and Depression*

The present project is a continuation of our efforts to understand mechanisms of depression as a comorbidity of epilepsy. The goal of this project is to explore central noradrenergic dysfunction as a mechanism of depression linked to epilepsy in those subjects in which serotonergic transmission is not primarily compromised.

R01 MD009149 (Hensler, PI)                      07/01/2014-06/30/2019  
Role PI, 2.4 calendar  
NIH                      \$250,000 Annual Direct Costs

*BDNF modulation of kynurenine pathway metabolism: neuroprotection during stress*

Exposure to trauma or stress has been shown to be one of the main predisposing risk factors to psychiatric disorders. However, many individuals exposed to adversity maintain normal psychological functioning, and the factors underlying resistance to the deleterious effects of stress remain unknown. We propose that BDNF functions as a modulator of kynurenine metabolism in brain, and that it is through this mechanism that BDNF confers resilience to stress. The proposed studies include experiments at the cellular, systems and behavioral level utilizing two mouse models: BDNF heterozygous mice (BDNF<sup>+/-</sup> mice), which exhibit marked reductions in BDNF expression; transgenic mice carrying the met allele of the human *bdnf* gene (BDNF<sub>met</sub> knock-in mice).

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Yes, changes affecting key personnel effort on this project have changed since the last reporting period. There was no research assistant following Corey Smolik's departure from 8/2015 – 3/2015 when Sergey Poplyayev was hired, and then overlap in research assistant effort with Marshall Edwards being hired from May – June until Sergey resigned. At the time of the original project end date, 9/29/2015 effort and salary coverage for Drs. Daws, Javors, Hensler and Koek ended, although Dr. Javors continues to perform measurements of D-22 for this project. Dr. Gould and Marshall Edwards each continue to work on the project with a 1.2 calendar months of effort from 9/29/2015 until the new project end date of 3/31/2016.

**Partner Organizations**

None to report

## REFERENCES CITED

Daws LC, Koek W, Mitchell NC. 2013. Revisiting serotonin reuptake inhibitors and the therapeutic potential of "uptake-2" in psychiatric disorders. *ACS Chem Neurosci*. 4:16-21.

Derosa G, Maffioli P. 2014. Alkaloids in the nature: pharmacological applications in clinical practice of berberine and mate tea. *Curr Top Med Chem*. 14,200-6.

Horton RE, Apple DM, Owens WA, Baganz NL, Cano S, Mitchell NC, Vitela M, Gould GG, Koek W, Daws LC. 2013. Decynium-22 enhances SSRI-induced antidepressant-like effects in mice: uncovering novel targets to treat depression. *J Neurosci*. 33:10534-43.

Lee N, Duan H, Hebert MF, Liang CJ, Rice KM, Wang J. Taste of a pill: organic cation transporter-3 (OCT3) mediates metformin accumulation and secretion in salivary glands. *J Biol Chem*. 2014 Sep 26;289(39):27055-64. doi:10.1074/jbc.M114.570564.

Reeves GM, Keeton C, Correll CU, Johnson JL, Hamer RM, Sikich L, Hazzard L, et al. 2013. Improving metabolic parameters of antipsychotic child treatment (IMPACT) study: rationale, design, and methods. *Child Adolesc Psychiatry Ment Health*. 7:31. doi: 10.1186/1753-2000-7-31.1.

Sun S, Wang K, Lei H, Li L, Tu M, Zeng S, Zhou H, Jiang H. 2014. Inhibition of organic cation transporter 2 and 3 may be involved in the mechanism of the antidepressant-like action of berberine. *Prog Neuropsychopharmacol Biol Psychiatry*. 49:1-6.

## APPENDICES:

- A. IACUC protocol related correspondence
- B. Amended contract with no cost extension
- C. Manuscript under review
- D. Poster presentation of berberine results from Mikiten Forum
- E. Symposium Description for "Serotonin in Seattle".

Appendix A. IACUC protocol related correspondence

January 16, 2015

To: Georgianna Gould, Ph.D. ([gouldg@uthscsa.edu](mailto:gouldg@uthscsa.edu))  
 Lynette Daws ([daws@uthscsa.edu](mailto:daws@uthscsa.edu))  
 UTHSCSA

Cc: Andrea Giuffrida, Ph.D., Institutional Official / Interim Vice President for Research  
 AVPRO ([schmelz@uthscsa.edu](mailto:schmelz@uthscsa.edu))  
 Department Chair Manzoor Bhat ([bhatm@uthscsa.edu](mailto:bhatm@uthscsa.edu))  
 AAALAC ([accredit@aaalac.org](mailto:accredit@aaalac.org))  
[kbayne@aaalac.org](mailto:kbayne@aaalac.org)

From: Institutional Animal Care and Use Committee (IACUC)

Subject: **Full Committee Stipulations of Noncompliance**

<b>Protocol Number: 12069x</b> <b>Title: Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders</b>
---

Dear Principal Investigator,

Your Noncompliance, dated January 8, 2015, for the above referenced protocol was reviewed by the Institutional Animal Care and Use Committee on January 14, 2015.

**Summary of Event:** Investigator continually violates the rodent housing policy by maintaining overcrowded cages to include multiple litters with different aged offspring.

**Summary of actions taken by PI, IACUC or institutional officials prior to IACUC meeting:** None

**Determination/IACUC Directed Actions:** The committee determined that the repeated incidences of overcrowded cages indicated a continuous failure to follow DLAR rodent housing policy and that the event meets the criteria for continuing noncompliance.

The Committee directed the PI to implement the following additional corrective actions by the next convened IACUC meeting:

- Provide the IACUC with written documentation for why cage overcrowding continues to occur.
- Provide the IACUC with an existing SOP or establish an SOP for laboratory personnel to follow on proper animal colony management in order to avoid overcrowding of animals in the future.
- PI and personnel must complete animal housing/colony management training with Dr. Nicole Nemetz

**Reporting:** Institutional Official, AVPRO, Department Chair, AAALAC International

The stipulations must be addressed promptly to complete the approval process. Please send your **point-by-point** response to the Committee stipulations (listed above) along with any applicable revised documents (with **tracked changes**) to [IACP@uthscsa.edu](mailto:IACP@uthscsa.edu).

**Please reply to this letter within 30 days of the IACUC meeting date noted above.**

<b>Sponsor's Name</b>	<b>Grant Title</b>	<b>OSP Number</b>
Department of Defense	Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders	153353

Sincerely,



Digitally signed by Patricia Alexander  
 DN: cn=Patricia Alexander, o=IRB,  
 ou=UTHSCSA,  
 email=alexanderp@uthscsa.edu, c=US  
 Reason: I have signed this letter  
 Date: 2015.01.22 14:50:12 -0600

Research Compliance Coordinator

*Please retain this document in your IACUC correspondence file*

<b>Institutional Animal Care Program</b>	Mail Code 7822	7703 Floyd Curl Drive	San Antonio, Texas 78229-3900
210.567.8260	Fax 210.567.8242	<a href="http://research.uthscsa.edu/iacuc">http://research.uthscsa.edu/iacuc</a>	

January 16, 2015 [Revised 02/11/15 to add DoD- Contact information]

To: Georgianna Gould, Ph.D. ([gouldg@uthscsa.edu](mailto:gouldg@uthscsa.edu))  
Lynette Daws ([daws@uthscsa.edu](mailto:daws@uthscsa.edu))  
UTHSCSA

Cc: Andrea Giuffrida, Ph.D., Institutional Official / Interim Vice President for Research  
AVPRO ([schmelz@uthscsa.edu](mailto:schmelz@uthscsa.edu))  
Department Chair Manzoor Bhat ([bhatm@uthscsa.edu](mailto:bhatm@uthscsa.edu))  
AAALAC ([accredit@aaalac.org](mailto:accredit@aaalac.org))  
[kbayne@aaalac.org](mailto:kbayne@aaalac.org)  
Department of Defense- Animal Care and Use Review Office ([acuro@amedd.army.mil](mailto:acuro@amedd.army.mil))

From: Institutional Animal Care and Use Committee (IACUC)

Subject: **Full Committee Stipulations of Noncompliance**

**Protocol Number: 12069x**

**Title: Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders**

Dear Principal Investigator,

Your Noncompliance, dated January 8, 2015, for the above referenced protocol was reviewed by the Institutional Animal Care and Use Committee on January 14, 2015.

**Summary of Event:** Investigator continually violates the rodent housing policy by maintaining overcrowded cages to include multiple litters with different aged offspring.

**Summary of actions taken by PI, IACUC or institutional officials prior to IACUC meeting:** None

**Determination/IACUC Directed Actions:** The committee determined that the repeated incidences of overcrowded cages indicated a continuous failure to follow DLAR rodent housing policy and that the event meets the criteria for continuing noncompliance.

The Committee directed the PI to implement the following additional corrective actions by the next convened IACUC meeting:

- Provide the IACUC with written documentation for why cage overcrowding continues to occur.
- Provide the IACUC with an existing SOP or establish an SOP for laboratory personnel to follow on proper animal colony management in order to avoid overcrowding of animals in the future.
- PI and personnel must complete animal housing/colony management training with Dr. Nicole Nemetz

**Reporting:** Institutional Official, AVPRO, Department Chair, AAALAC International, DoD

The stipulations must be addressed promptly to complete the approval process. Please send your **point-by-point** response to the Committee stipulations (listed above) along with any applicable revised documents (with **tracked changes**) to [IACP@uthscsa.edu](mailto:IACP@uthscsa.edu).

**Please reply to this letter within 30 days of the IACUC meeting date noted above.**

<b>Sponsor's Name</b>	<b>Grant Title</b>	<b>OSP Number</b>
Department of Defense	Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders	153353

Sincerely,

Research Compliance Coordinator

*Please retain this document in your IACUC correspondence file*

**Institutional Animal Care Program** | Mail Code 7822 | 7703 Floyd Curl Drive | San Antonio, Texas 78229-3900

210.567.8260 | Fax 210.567.8242 | <http://research.uthscsa.edu/iacuc>

IACP-7000

**February 17, 2015**

**To:** Patricia Alexander, Research Regulatory Programs, Institutional Review Board([AlexanderP@uthscsa.edu](mailto:AlexanderP@uthscsa.edu))  
Institutional Care and Use Committee ([iacp@uthscsa.edu](mailto:iacp@uthscsa.edu))

**From:** Georgianna Gould

**Subject:** Full Committee Stipulations of Non-Compliance for Protocol 12069x

To All Concerned,

I sincerely apologize if there was any non-compliance regarding overcrowded mice on this protocol. Since the alleged offenses, which per LAR personnel occurred between 11/15/2014 and 1/6/2015, I raise the following counter-arguments and/or have taken the following measures to remedy the situation:

1. I attended a meeting on 1/28/2015 for **additional animal housing/colony management training with Dr. Nicole Nemetz** at 8:30 am in room 1.014V, the animal colony housing room. During that training meeting, we discussed how breeding and development of my BTBR mice differs from other commonly used strains. She suggested that I could apply for protocol modification to include exemptions to allow for cross-fostering with multiple nursing females in the same cage to enhance pup survival, and to extend the age of weaning to an apparent 28 days by weight to allow for the BTBR to reach the appropriate age for weaning (at 21 days):
  - a. In November, an LAR attendant was newly assigned to my housing room and colony, and they were unfamiliar with the reproductive capacity and postnatal development of the inbred BTBR mice in question. For the record, BTBR mice have litters that average 12 pups, which are larger than 3-6 pups/litter typical of more commonly used strains, such C57BL/6J. The new LAR attendant was not aware that cross fostering could enhance BTBR pup survival. Survivorship and health of BTBR pups from large litters born to new mothers is greatly enhanced by having a veteran nursing BTBR mother whose prior litters had good survivorship in the cage to cross-foster. Often first litters born to BTBR are eaten or not nursed by the mother, but this is less likely to happen when a more experienced nursing dam is present. **Given this, and following consultation with Dr. Nemetz, I submitted a formal request to IACP for an IACUC protocol modification for exemption to allow for two litters per cage to allow for cross-fostering and enhanced pup survival.**
  - b. While BTBR mice grow more quickly and are heavier than C57BL/6 prior to weaning, they still should not be weaned prior to postnatal 21 days. Some of the cages flagged as overcrowded had litters less than 21 days old. To comply with LAR personnel's requests, I have separated BTBR mice as young as 16 days old. The outcome of premature weaning was shivering pups with slower development, distended bellies that didn't groom themselves. Given that behavioral measures are perhaps the most critical endpoint, I remain concerned that continuing such practices will compromise our studies. **Hence, some of the offenses cited were not actual incidences of overcrowding, but were due instead to higher weights of juvenile BTBR mice in contrast to other strains.** However, given that few investigators use these mice, I also included in my request for a protocol modification **a request for exemption to extend weaning to 28 days**, since BTBR mice at 21 days have comparable weights and body sizes to C57BL/6 mice at 28 days.
2. **Hiring of a Laboratory Technician**, Sergey Poplyayev was interviewed 2/9/14 and human resources is processing his hire before the close of February 2015. Once he is working and completes CITI and LAR training, his primary responsibility will be to maintain and monitor my mouse colonies and to prevent cage overcrowding.

3. As requested the following is provided to address “**why cage overcrowding continues to occur**”:

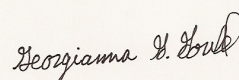
- a. With all due respect, I contend that the allegation that I as the PI took no measures to remediate the noncompliance between 12/28/15 and 1/14/15 is an overstatement. **Before I was even aware that a report had been filed, I separated many overcrowded cages, but these measures I took were not included in the record presented to the IACUC.**
- b. In early November I stepped up breeding to assure that age-matched adult male mice will be available for spring and summer experiments. Given that mouse pregnancy onset varied over two weeks based on the timing of matings, for about 2 months as the resulting pups grew and matured, I separated cages as they became overcrowded, but each day new and different cages became overcrowded, and were separated as quickly as possible. Hence this was not neglect of a single cage, but instead was a planned ongoing process in response to the need for future studies as evidenced by my continued submission of requests to euthanize weaned females and retired breeders. **It appears the complaint mistakenly assumes the same cages with the same mice remained overcrowded for many days, but this was not actually the case.**
- c. Between travel to a meeting and a family health care crisis in December-early January, there were two 2-3 day periods wherein I was not on campus, and Alicia Sanchez did not come in to work, and overcrowded cages were left for >48 hours. Personnel in Dr. Daws’ laboratory designated on the IACUC protocol at the time as working with these mice (specifically Melissa Vitela and William Anthony Owens) were not contacted by LAR and asked to separate the overcrowded cages. While our grants administrator Ludmila Grinshpan was notified on one occasion in November, she does not work with the mice, but only handles paperwork. **Since that time, Melissa will be replaced by Sergey on the protocol, but the contact information for personnel on the IACUC protocol designated to handle mice should be made available to LAR personnel.**
- d. To prepare for the help I knew would be needed with the stepped-up breeding occurring in November and December, on November 6, 2014, **I initiated the process of hiring a technician to maintain the mice through my department, but the hiring process took much longer than anticipated, despite my best efforts.**

4. Finally, as required, **I establish an SOP for laboratory personnel to follow in cage density:**

- a. Monitor mice in the colony every morning and separate cages with the potential to become overcrowded before they do and are flagged by LAR personnel.
- b. Separate pregnant mice prior to giving birth unless or until an exemption has been approved that excludes the need to do so in the case of cross-fostering to enhance pup survivorship.
- c. Separate mouse pups from mothers and siblings to a density of 5 mice per cage as soon as they appear to be 21 days old based on their size (not actual age) unless or until an exemption has been approved that excludes the need to do so until they appear to be 28 days of age.
- d. If there are any concerns about a cage that has been flagged by LAR personnel as overcrowded in which pups are too young to be weaned, please contact Dr. Nicole Nemetz and she will advise LAR personnel to delay as necessary before requiring their separation.

The measures taken and underway should prevent overcrowded cage violations from occurring.

Sincerely,



May 28, 2015

To: Georgianna Gould, Ph.D. ([gouldg@uthscsa.edu](mailto:gouldg@uthscsa.edu))  
UTHSCSA

From: Institutional Animal Care and Use Committee (IACUC)

Subject: **Approval of a Response to IACUC Stipulations for a Full Committee Review of Possible Noncompliance**

<b>Protocol Number: 12069x</b> <b>Title: Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders</b>
---

Dear Principal Investigator,

All of the specified conditions for approval which were stipulated by Institutional Animal Care and Use Committee (IACUC) on January 14, 2015 and February 25, 2015 have been met. Final approval was given on May 20, 2015.

The IACUC determined the PI has satisfied the corrective action plan noted in the stipulations and that the submitted amendment will be reviewed by the full committee at a subsequent meeting.

Be sure to summarize this report of noncompliance in your next progress report.

<b><i>Sponsor's Name</i></b>	<b><i>Grant Title</i></b>	<b><i>OSP Number</i></b>
<i>Department of Defense</i>	<i>Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders</i>	<i>153353</i>

My point of contact on this issue is Dr. Kimberly Summers; Director, Research Regulatory Programs.

Sincerely,

Staff Signature Block  
IACP Staff

*Please retain this document in your IACUC correspondence file*

<b>Institutional Animal Care Program</b>	Mail Code 7822	7703 Floyd Curl Drive	San Antonio, Texas 78229-3900
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210.567.8260 | Fax 210.567.8242 | <http://research.uthscsa.edu/iacuc>



August 28, 2015

To: Georgianna Gould, Ph.D.  
Lynette C. Daws, Ph.D. (daws@uthscsa.edu)  
Lyudmila Grinshpan, M.S. ([grinshpan@uthscsa.edu](mailto:grinshpan@uthscsa.edu))  
Physiology, MC 7764  
UTHSCSA

From: Institutional Animal Care and Use Committee (IACUC)

Subject: **Approval of a Modification to an Ongoing Protocol (Amendment) – Designated Review**

<b>Protocol Number: 12069x</b> <b>Title: Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders</b>
---

Dear Principal Investigator,

Your request to modify this protocol (amendment), dated August 7, 2015, was reviewed by Designated Review on August 27, 2015 and approved.

Summary of the main modifications to your protocol: Increase animal numbers.

The approval of this modification does not change the IACUC expiration date. **IACUC Expiration Date:** August 21, 2015.

<i><b>Sponsor's Name</b></i>	<i><b>Grant Title</b></i>	<i><b>OSP Number</b></i>
Department of Defense	Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders	153353

This Institution has an Animal Welfare Assurance on file with the NIH Office of Laboratory Animal Welfare. The Assurance Number is A3345-01. The care and use of animals is in accordance with the NRC Publication, as revised in 2011, "Guide for the Care and Use of Laboratory Animals," and other applicable Federal regulations.

Sincerely,

Research Compliance Specialist  
Research Regulatory Programs

*Please retain this document in your IACUC correspondence file*

<b>Institutional Animal Care Program</b>	Mail Code 7822	7703 Floyd Curl Drive	San Antonio, Texas 78229-3900
	210.567.8260	Fax 210.567.8242	<a href="http://research.uthscsa.edu/iacuc">http://research.uthscsa.edu/iacuc</a>

September 4, 2015

To: Georgianna Gould, Ph.D.  
Lynette C. Daws, Ph.D. (daws@uthscsa.edu)  
Lyudmila Grinshpan, M.S. ([grinshpan@uthscsa.edu](mailto:grinshpan@uthscsa.edu))  
Physiology, MC 7764  
UTHSCSA

From: Institutional Animal Care and Use Committee (IACUC)

Subject: **Re-approval of an Animal Use Protocol – Designated Review**

<p align="center"><b>Protocol Number: 12069x</b> <b>Title: Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders</b></p>
--

Dear Principal Investigator,

Your progress report, dated August 7, 2015, was reviewed and approved by Designated Review on September 3, 2015. Your protocol has been approved to continue.

**New Protocol Expiration Date:** August 21, 2016.

Your next progress report must be submitted to the IACP Office 30 days before the protocol expiration date.

<i><b>Sponsor's Name</b></i>	<i><b>Grant Title</b></i>	<i><b>OSP Number</b></i>
Department of Defense	Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders	153353

This Institution has an Animal Welfare Assurance on file with the NIH Office of Laboratory Animal Welfare. The Assurance Number is A3345-01. The care and use of animals is in accordance with the NRC Publication, as revised in 2011, "Guide for the Care and Use of Laboratory Animals," and other applicable Federal regulations.

Sincerely,

Research Compliance Specialist  
Research Regulatory Programs

*Please retain this document in your IACUC correspondence file*

<b>Institutional Animal Care Program</b>	Mail Code 7822	7703 Floyd Curl Drive	San Antonio, Texas 78229-3900
	210.567.8260	Fax 210.567.8242	<a href="http://research.uthscsa.edu/iacuc">http://research.uthscsa.edu/iacuc</a>

## Appendix B. Amended contract with no cost extension

<b>AMENDMENT OF SOLICITATION/MODIFICATION OF CONTRACT</b>				1. CONTRACT ID CODE <b>S</b>		PAGE OF PAGES <b>1   12</b>	
2. AMENDMENT/MODIFICATION NO. <b>P00001</b>		3. EFFECTIVE DATE <b>14-Aug-2015</b>		4. REQUISITION/PURCHASE REQ. NO. <b>W91ZSQ2105N646</b>		5. PROJECT NO.(If applicable)	
6. ISSUED BY USA MED RESEARCH ACQ ACTIVITY 820 CHANDLER ST FORT DETRICK MD 21702-5014		CODE <b>W81XWH</b>		7. ADMINISTERED BY (If other than item 6)  <b>See Item 6</b>		CODE	
8. NAME AND ADDRESS OF CONTRACTOR (No., Street, County, State and Zip Code) UNIVERSITY OF TEXAS HEALTH SCIENCE CENTE OFFICE OF GRANTS MANAGEMENT 7703 FLOYD CURL DR SAN ANTONIO TX 78229-3901				9A. AMENDMENT OF SOLICITATION NO.			
				9B. DATED (SEE ITEM 11)			
				X 10A. MOD. OF CONTRACT/ORDER NO. <b>W81XWH-12-1-0506</b>			
				X 10B. DATED (SEE ITEM 13) <b>30-Sep-2012</b>			
CODE <b>0NJ12</b>		FACILITY CODE					
11. THIS ITEM ONLY APPLIES TO AMENDMENTS OF SOLICITATIONS							
<input type="checkbox"/> The above numbered solicitation is amended as set forth in Item 14. The hour and date specified for receipt of Offer <input type="checkbox"/> is extended, <input type="checkbox"/> is not extended. Offer must acknowledge receipt of this amendment prior to the hour and date specified in the solicitation or as amended by one of the following methods: (a) By completing Items 8 and 15, and returning _____ copies of the amendment; (b) By acknowledging receipt of this amendment on each copy of the offer submitted; or (c) By separate letter or telegram which includes a reference to the solicitation and amendment numbers. FAILURE OF YOUR ACKNOWLEDGMENT TO BE RECEIVED AT THE PLACE DESIGNATED FOR THE RECEIPT OF OFFERS PRIOR TO THE HOUR AND DATE SPECIFIED MAY RESULT IN REJECTION OF YOUR OFFER. If by virtue of this amendment you desire to change an offer already submitted, such change may be made by telegram or letter, provided each telegram or letter makes reference to the solicitation and this amendment, and is received prior to the opening hour and date specified.							
12. ACCOUNTING AND APPROPRIATION DATA (If required)							
13. THIS ITEM APPLIES ONLY TO MODIFICATIONS OF CONTRACTS/ORDERS. IT MODIFIES THE CONTRACT/ORDER NO. AS DESCRIBED IN ITEM 14.							
A. THIS CHANGE ORDER IS ISSUED PURSUANT TO: (Specify authority) THE CHANGES SET FORTH IN ITEM 14 ARE MADE IN THE CONTRACT ORDER NO. IN ITEM 10A.							
B. THE ABOVE NUMBERED CONTRACT/ORDER IS MODIFIED TO REFLECT THE ADMINISTRATIVE CHANGES (such as changes in paying office, appropriation date, etc.) SET FORTH IN ITEM 14, PURSUANT TO THE AUTHORITY OF FAR 43.103(B).							
C. THIS SUPPLEMENTAL AGREEMENT IS ENTERED INTO PURSUANT TO AUTHORITY OF:							
X D. OTHER (Specify type of modification and authority) <b>USAMRAA Terms &amp; Conditions, reference letter request dated 11 Aug 2015</b>							
E. IMPORTANT: Contractor <input checked="" type="checkbox"/> is not, <input type="checkbox"/> is required to sign this document and return _____ copies to the issuing office.							
14. DESCRIPTION OF AMENDMENT/MODIFICATION (Organized by UCF section headings, including solicitation/contract subject matter where feasible.) Modification Control Number: <b>ebane1155052</b> Principal Investigator: <b>Dr. Georgianna Gould</b> Proposal Title: <b>"Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders"</b> Period of Performance: <b>30 September 2012 - 31 March 2016</b> Total Award and Funded Amount: <b>\$560,625</b>  The purpose of this modification is to: (1) provide a 6-months no-cost extension (NCE) to the Period of Performance for CLIN 0001, extending the end date to 31 March 2016; and (2) make administrative changes to the Award General Terms and Conditions (see highlighted areas). The final technical report is due no later than 29 June 2016. Submissions of financial reports (SF425s) shall continue during the NCE period. <b>See SUMMARY of CHANGES</b>							
Except as provided herein, all terms and conditions of the document referenced in Item 9A or 10A, as heretofore changed, remains unchanged and in full force and effect.							
15A. NAME AND TITLE OF SIGNER (Type or print)				16A. NAME AND TITLE OF CONTRACTING OFFICER (Type or print) CHRISTINE HELMAN / CONTRACTING OFFICER TEL: 301-619-2265 EMAIL: christine.e.helman.civ@mail.mil			
15B. CONTRACTOR/OFFEROR  (Signature of person authorized to sign)		15C. DATE SIGNED		16B. UNITED STATES OF AMERICA BY <u>Christine E. Helman</u> (Signature of Contracting Officer)		16C. DATE SIGNED <b>14-Aug-2015</b>	

## SECTION SF 30 BLOCK 14 CONTINUATION PAGE

**SUMMARY OF CHANGES**

## SECTION 00010 - SOLICITATION CONTRACT FORM

The standard size code 500 has been added.

The NAICS code 541712 has been added.

The 'administered by' organization has changed from

US ARMY MEDICAL RESEARCH ACQUISITION ACT

ATTN: LISA ZARICK

301-619-2336

LISA.ZARICK@AMEDD.ARMY.MIL

FORT DETRICK MD 21702

to

USA MED RESEARCH ACQ ACTIVITY

820 CHANDLER ST

FORT DETRICK MD 21702-5014

## CLIN 0001

The CLIN extended description has changed from:

Idea Development Award. PERIOD OF PERFORMANCE: 30 September 2012 – 29 September 2015

To:

Idea Development Award. PERIOD OF PERFORMANCE: 30 September 2012 – 31 March 2016.

## DELIVERIES AND PERFORMANCE

The following Delivery Schedule item for CLIN 0001 has been changed from:

DELIVERY DATE	QUANTITY	SHIP TO ADDRESS	DODAAC
POP 30-SEP-2012 TO 29-SEP-2015	N/A	USA MED RESEARCH MAT CMD 1077 PATCHEL STREET BLDG 1077 FORT DETRICK MD 21702 FOB: Destination	W91ZSQ

To:

DELIVERY DATE	QUANTITY	SHIP TO ADDRESS	DODAAC
POP 30-SEP-2012 TO 31-MAR-2016	N/A	USA MED RESEARCH MAT CMD 1077 PATCHEL STREET BLDG 1077 FORT DETRICK MD 21702 FOB: Destination	W91ZSQ

The following have been modified:

PI NAME & PROPOSAL TITLE

**PRINCIPAL INVESTIGATOR:** Dr. Georgianna Gould

**TITLE:** “Novel Therapeutic Targets To Treat Social Behavior Deficits in Autism and Related Disorders”

**Administered By:**

Elena Howell  
Grants Specialist  
US Army Medical Research Acquisition Activity  
Phone: 301-619-6871  
Email: [Elena.g.howell.civ@mail.mil](mailto:Elena.g.howell.civ@mail.mil)

SECTION 00800 - SPECIAL CONTRACT REQUIREMENTS

The following have been modified:

**TERMS AND CONDITIONS**

**U.S. ARMY MEDICAL RESEARCH AND MATERIEL COMMAND (USAMRMC)**

**U.S. ARMY MEDICAL RESEARCH ACQUISITION ACTIVITY (USAMRAA)**

**Effective October 1, 2011**

**TERMS AND CONDITIONS INCORPORATED BY REFERENCE (APRIL 2012)**

The recipient shall comply with the terms and conditions below that are applicable to its type of organization:

a. **For Educational and Non-Profit Organizations:** This award incorporates by reference, with the same force and effect as if they were included in full text, the Research Terms and Conditions dated June 2011 (<http://www.nsf.gov/awards/managing/rtc.jsp>) and the USAMRAA Agency Specific Requirements dated October 1, 2011 (<https://www.usamraa.army.mil>).

b. **For Commercial (For-Profit) Organizations:** This award incorporates by reference, with the same force and effect as if they were included in full text, the USAMRAA General Terms and Conditions for Assistance Awards with For-Profit Organizations dated April 1, 2012 (<https://www.usamraa.army.mil>).

Any apparent inconsistency between Federal statutes and regulations and the terms and conditions contained in this award shall be referred to the USAMRAA Contract/Grants Specialist for guidance.

**AWARD SPECIFIC TERMS AND CONDITIONS**

This award is made under the authority of 31 U.S.C. 6304 and 10 U.S.C. 2358. The recipient's revised Statement of Work (SOW) dated 6 July 2012 and the revised budget dated 25 July 2012 for the application submitted in response to the Fiscal Year 11 Department of Defense (DOD) Autism Research Program Idea Development Award Announcement (Funding Opportunity Number: W81XWH-11-ARP-IDA), which closed 30 November 2011 are incorporated herein by reference. The Catalog of Federal Domestic Assistance Number relative to this award is CFDA 12.420.

## ACCEPTANCE OF AWARD

The recipient is not required to countersign this award. In case of disagreement, the recipient shall notify the USAMRAA Grants Officer and not assess the award any costs until such disagreement(s) is resolved.

## ADMINISTRATIVE AND COST PRINCIPLES

The following Administrative and Cost Principles, as applicable, effective the earlier of (i) the start date of this award or (ii) the date on which the recipient incurs costs to be assessed against the award, are incorporated as part of this award by reference:

- a. CFR, Title 2, Part 220, "Cost Principles for Educational Institutions (OMB Circular A-21)."
- b. CFR, Title 2, Part 225, "Cost Principles for State, Local, and Indian Tribal Governments (OMB Circular A-87)."
- c. OMB Circular A-102, "Grants and Cooperative Agreements with State and Local Governments."
- d. CFR, Title 2, Part 215, "Uniform Administrative Requirements for Grants and Agreements with Institutions of Higher Education, Hospitals, and Other Non-profit Organizations (OMB Circular A-110)."
- e. CFR, Title 2, Part 230, "Cost Principles for Non-profit Organizations (OMB Circular A-122)." [For those nonprofit organizations specifically excluded from the provisions of OMB Circular A-122, Subpart 31.2 of the Federal Acquisition Regulations (FAR 48 CFR Subpart 31.2) shall apply].
- f. OMB Circular A-133, "Audits of States, Local Governments, and Non-Profit Organizations."
- g. Federal Acquisition Regulation, Part 31.2, for Commercial Organizations and those nonprofit organizations specifically excluded from the provisions of OMB Circular A-122.
- h. Department of Defense Grant and Agreement Regulations 3210.6-R.

These publications may be obtained from:

Office of Management and Budget  
EOP Publications Office  
New Executive Office Building  
725 17<sup>th</sup> Street, NW, Room 2200  
Washington, DC 20503  
Telephone: (202) 395-7332  
Website: <http://www.whitehouse.gov/omb/>

## RECIPIENT RESPONSIBILITY

In addition to the responsibilities of the recipient as defined in the award or incorporated by reference herein:

- a. The recipient will bear primary responsibility for the conduct of the research and will exercise sound judgment within the limits of the award's terms and conditions.
- b. The Principal Investigator(s) (PI) specified in the award document will be continuously responsible for the conduct of the research project and will be closely involved with the research effort. The PI, operating within the policies of the recipient, is in the best position to determine the means by which the research may be conducted most effectively.

c. The recipient shall request the USAMRAA Grants Officer's prior approval to change the PI or any key personnel, for the PI or any key personnel to be absent from the project during any continuous period of 3 months or more, or for the PI or any key personnel to reduce time devoted to the project by 25 percent or more from the level that was approved at the time of award.

## **AWARD MODIFICATION**

The only method by which this award may be modified is by a formal, written modification signed by the USAMRAA Grants Officer. No other communications, whether oral or in writing, are valid to change the terms and conditions of this award. See the USAMRAA Agency Specific Requirements for changes requiring USAMRAA Grants Officer's prior approval.

## **MAXIMUM OBLIGATION**

The maximum obligation of the Government for support of this award will not exceed the amount specified in the award, as modified. Awards will not be modified to provide additional funds for such purposes as reimbursement for unrecovered indirect costs resulting from the establishment of final negotiated rates or for increases in salaries, fringe benefits, and other costs.

## **SUPPORTING INFORMATION**

Information such as subawards, consultant agreements, vendor quotes, and personnel work agreements may be required in order to support proposed costs or to determine the employment status of personnel. The Government's receipt of this information does not constitute approval or acceptance of any term or condition included therein.

## **FINANCIAL INSTABILITY, INSOLVENCY, BANKRUPTCY OR RECEIVERSHIP**

a. The recipient shall immediately notify the USAMRAA Grants Officer of the occurrence of the following events: (1) the recipient's financial instability that would negatively impact performance of this award; (2) the recipient's or recipient's parent's filing of a voluntary case seeking liquidation or reorganization under the Bankruptcy Act; (3) the recipient's consent to the institution of an involuntary case under the Bankruptcy Act against the organization or organization's parent; (4) the filing of any similar proceeding for or against the recipient or recipient's parent, or its consent to, the dissolution, winding-up or readjustment of the recipient's debts, appointment of a receiver, conservator, trustee, or other officer with similar powers over the organization, under any other applicable state or federal law; or (5) the recipient's insolvency due to its inability to pay its debts generally as they become due.

b. Such notification shall be in writing and shall: (1) specifically set out the details of the occurrence of an event referenced in paragraph a; (2) provide the facts surrounding that event; and (3) provide the impact such event will have on the project being funded by this award.

c. Upon the occurrence of any of the five events described in the first paragraph, the Government reserves the right to conduct a review of this award to determine the recipient's compliance with the required elements of the award (including such items as cost share, progress towards technical project objectives, and submission of required reports). If the USAMRAA Grants Officer's review determines that there are significant deficiencies or concerns with the recipient's performance under the award, the Government reserves the right to impose additional requirements, as needed, including (1) change the payment method; (2) institute payment controls, and (3) require additional reporting requirements.

d. Failure of the recipient to comply with this term may be considered a material failure by the recipient to comply with the terms of this award and may result in termination.



**PROHIBITION OF USE OF LABORATORY ANIMALS****\*\* PROHIBITION – READ FURTHER FOR DETAILS \*\***

Notwithstanding any other terms and conditions contained in this award or incorporated by reference herein, the recipient is expressly forbidden to use or subcontract for the use of laboratory animals in any manner whatsoever without the express written approval of the USAMRMC, Animal Care and Use Review Office (ACURO). Written authorization to begin research under the applicable protocol(s) proposed for this award will be issued in the form of an approval letter from the USAMRMC ACURO to the recipient. Furthermore, modifications to already approved protocols require approval by ACURO prior to implementation. For each fiscal year, the recipient shall maintain, and upon request from ACURO, submit animal usage information.

Non-compliance with any of these terms and conditions may result in withholding of funds and/or the termination of the award.

**PROHIBITION OF USE OF HUMAN SUBJECTS****\*\* PROHIBITION – READ FURTHER FOR DETAILS \*\***

Research under this award involving the use of human subjects, to include the use of human anatomical substances or identifiable private information, shall not begin until the USAMRMC's Office of Research Protections (ORP) provides authorization that the research may proceed. Written approval to begin research will be issued from the USAMRMC ORP, under separate notification to the recipient. Written approval from the USAMRMC ORP is also required for any subrecipient that will use funds from this award to conduct research involving human subjects.

Research involving human subjects shall be conducted in accordance with the protocol submitted to and approved by the USAMRMC ORP. Complete study records shall be maintained for each human research study and shall be made available for review by representatives of the USAMRMC. Research records shall be stored in a manner so as to protect the confidentiality of subject information.

The recipient is required to adhere to the following reporting requirements:

Submission of major modifications to the protocol, continuing review documentation, and the final report are required as outlined in the USAMRMC ORP approval memorandum.

Unanticipated problems involving risks to subjects or others, subject deaths related to participation in the research, clinical holds (voluntary or involuntary), and suspension or termination of this research by the IRB, the institution, the Sponsor, or regulatory agencies, shall be promptly reported to the USAMRMC ORP.

The knowledge of any pending compliance inspection/visits by the FDA, ORP, or other government agency concerning this clinical investigation or research, the issuance of Inspection Reports, FDA Form 483, warning letters or actions taken by any Regulatory Agencies including legal or medical actions, and any instances of serious or continuing noncompliance with regulatory requirements that relate to this clinical investigation or research, shall be reported immediately to the USAMRMC ORP.

Non-compliance with these terms and conditions may result in withholding of funds and/or the termination of the award.

**PROHIBITION OF USE OF HUMAN CADAVERS****\*\* PROHIBITION – READ FURTHER FOR DETAILS \*\***

Research, development, testing and evaluation (RDT&E), education or training activities involving human cadavers under this award shall not begin until approval is granted in accordance with the Army Policy for Use of Human Cadavers for RDT&E, Education, or Training, 20 April 2012

([https://mrmc.amedd.army.mil/index.cfm?pageid=research\\_protections.overview](https://mrmc.amedd.army.mil/index.cfm?pageid=research_protections.overview)). The USAMRMC Office of Research Protections (ORP) is the Action Office ([usarmy.detrick.medcom-usamrmc.other.hrpo@mail.mil](mailto:usarmy.detrick.medcom-usamrmc.other.hrpo@mail.mil)) for this policy. Written approvals to begin the activity will be issued under separate notification to the recipient. Noncompliance with these terms and conditions may result in withholding of funds and/or the termination of the award.

## PATENTS AND INVENTIONS REPORTING REQUIREMENTS

a. iEdison and annual reporting. The recipient shall electronically file Invention Disclosures and Patent Applications using the Interagency Edison (iEdison) system through the National Institutes of Health (<https://s-edison.info.nih.gov/iEdison>) within the times specified for reporting. In addition, inventions made during the year shall also be reported annually (within 30 days of the anniversary date of the award) on a DD Form 882, "Report of Inventions and Subcontracts." The report shall be sent electronically to [Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil](mailto:Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil). If there are no inventions during the year, no annual DD Form 882 is required. The DD Form 882 can be accessed at <https://www.usamraa.army.mil>.

b. Closeout report. A final DD Form 882 is required. The form shall be submitted electronically to [Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil](mailto:Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil) within 90 days of end of the term of award and shall list all inventions made during the term of the award, or state "none", as applicable. The award will NOT be closed until all reporting requirements have been met.

## FINANCIAL REPORTING REQUIREMENTS

The recipient shall use the Standard Form (SF) 425, "Federal Financial Report," for reporting individual awards. Quarterly and final reports are required for those awards receiving advance payments. Annual and final reports are required for those awards receiving cost reimbursement payments.

The Federal Financial Reporting period end dates fall on the end of the calendar quarter for quarterly reports (3/31, 6/30, 9/30, 12/31), end of the calendar year for annual reports (12/31), and the end date of the term of award for the final report. Quarterly reports shall be submitted no later than 30 days after the end of each quarter. Annual reports shall be submitted no later than 90 days after the end of the calendar year. Final reports shall be submitted no later than 90 days after the end date of the term of award.

### Submission Instructions:

- a. All SF425 reports must be submitted electronically through the web site <https://www.usamraa.army.mil/pages/sf425>. The form and instructions can be obtained on this site.
- b. Do not report multiple awards on one report. Each award must be reported separately on its own SF425.
- c. Do not combine multiple SF425s into one submission. Each form must be saved as a separate PDF and submitted individually.

## TECHNICAL REPORTING REQUIREMENTS

- a. Annual and Final Technical Reports

Annual and final technical reports are required. Submission of annual and final technical reports, in electronic format (PDF or Word file only), shall be submitted to <https://ers.amedd.army.mil>. Problems accessing this site should be brought to the attention of the USAMRMC Help Desk at 301-619-2049.

Annual reports shall provide a complete summary of the research accomplishments to date with respect to the approved SOW. Journal articles can be substituted for detailed descriptions of specific aspects of the research, but the original articles shall be attached to the report as an appendix and appropriately referenced in the text. The importance of the report to decisions relating to continued support of the research can not be over-

emphasized. An annual report shall be submitted within 30 calendar days of the anniversary date of the award for the preceding 12 month period. If the award period of performance is extended by the USAMRAA Grants Officer, an annual report is still required to be submitted within 30 days of the anniversary date of the award.

A final report summarizing the entire research effort, citing data in the annual reports and appended publications, shall be submitted within 90 days of the end of the term of award. The final report shall provide a complete reporting of the research findings. Journal publications can be substituted for detailed descriptions of specific aspects of the research, but an original copy of each publication shall be attached as an appendix and appropriately referenced in the text. The final report shall include a bibliography of all publications and meeting abstracts and a list of personnel receiving pay (do not include salaries/stipends) from the research effort.

#### b. Format Requirements

Format requirements are provided at

[https://mrnc.amedd.army.mil/index.cfm?pageid=researcher\\_resources.technical\\_reporting](https://mrnc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting).

Although there is no page limitation for the reports, each report shall be of sufficient length to provide a thorough description of the accomplishments with respect to the approved SOW. All reports shall have the following elements, in this order:

**FRONT COVER:** The Accession Document (AD) Number should remain blank.

**STANDARD FORM (SF) 298 "Report Documentation Page:"** The abstract in Block 14 shall state the purpose, scope, and major findings, and be an up-to-date report of the progress in terms of results and significance. Subject terms are keywords that may have been assigned to the abstract or are keywords that may be significant to the research. The number of pages shall include all pages that have printed data (including the front cover, SF298, table of contents, and all appendices). Count pages carefully to ensure legibility and that there are no missing pages, as this delays processing of reports. Page numbers shall be typed.

**TABLE OF CONTENTS:** The text of the report shall include all sections addressed in the table of contents, to include:

**INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose, and scope of the research.

**BODY:** This section of the report shall describe the research accomplishments associated with each task outlined in the approved SOW. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Provide data explaining the relationship of the most recent findings with that of previously reported findings. Appended publications and/or presentations may be substituted for detailed descriptions of methodology but shall be referenced in the body of the report. If applicable, for each task outlined in the SOW, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included. **However, changes to the original SOW shall be approved by the USAMRAA Grants Officer through an award modification prior to initiating any changes.**

**KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research.

**REPORTABLE OUTCOMES:** Provide a list of reportable outcomes that have resulted from this research, to include:

manuscripts, abstracts, presentations;  
patents and licenses applied for and/or issued;  
degrees obtained that are supported by this award;  
development of cell lines, tissue, or serum repositories;  
informatics such as databases and animal models, etc.;  
funding applied for based on work supported by this award;  
employment or research opportunities applied for and/or received based on experience/training supported by this award.

**CONCLUSION:** Summarize the results to include the importance and/or implications of the completed research and, when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included.

**REFERENCES:** List all references pertinent to the report using a standard journal format (i.e., format used in Science, Military Medicine, etc.).

**APPENDICES:** Attach all appendices that contain information that supplements, clarifies, or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, surveys, etc.

**SUPPORTING DATA:** All figures and/or tables shall include legends and be clearly marked with figure/table numbers.

Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.**

Mark all pages of the report which contain proprietary or unpublished data that should be protected by the U.S. Government. **REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE.** It is the responsibility of the PI to advise the USAMRMC when restricted limitation assigned to a document can be downgraded to Approved for Public Release. **DO NOT USE THE WORD " WHEN MARKING DOCUMENTS.**

## **DELINQUENT REPORTS**

If the recipient is delinquent on reporting requirements for other USAMRAA-sponsored awards, payments on this award may be withheld until acceptable delinquent reports have been submitted.

## **INVOICING FOR ADVANCE PAYMENTS WITH FULL FUNDING (JANUARY 2012)**

a. Payments. Advance payments will be made to the recipient upon receipt of an invoice submitted through Wide Area Work Flow (WAWF) in accordance with the Contract Line Item Number (CLIN) structure set forth in this award. It is anticipated that Defense Finance and Accounting Service (DFAS) will disburse funds within 30 days of receipt of a proper invoice.

b. **A copy of the most recently submitted Federal Financial Report (SF 425) shall be attached in WAWF and submitted with each invoice for all invoice submissions subsequent to the initial invoice submission.**

c. Electronic Funds Transfer (EFT). All payments will be made by EFT to the recipient's financial institution account listed in the System for Award Management (SAM) (located at <https://www.sam.gov>). Failure to update SAM and ensure your account is in an active status will result in nonpayment.

d. If the recipient fails to perform or if the WAWF invoice submission does not have the most recent SF425 attached, the invoice will be rejected.

e. Interest Bearing Account. Unless exempted by applicable Treasury-State agreements in accordance with the Cash Management Improvement Act (CMIA) (31 U.S.C. 3335), the recipient shall deposit all advance payments into an interest bearing account. Interest over the amount of \$250 per year shall be remitted annually to the U.S. Department of Health and Human Services, Payment Management System, P.O. Box 6021, Rockville, Maryland 20852. A copy of the transmittal letter stating the amount of interest remitted shall be sent electronically to [Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil](mailto:Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil).

f. Invoicing Schedule for Advances

Year One \$186,875.00

<u>Amount</u>	<u>Invoice Submission Date (on or about)</u>	<u>CLIN</u>
\$46,718.75	30 September 2012	0001
\$46,718.75	30 December 2012	0001
\$46,718.75	30 March 2013	0001
\$46,718.75	30 June 2013	0001

Year Two \$186,875.00

<u>Amount</u>	<u>Invoice Submission Date (on or about)</u>	<u>CLIN</u>
\$46,718.75	30 September 2013	0001
\$46,718.75	30 December 2013	0001
\$46,718.75	30 March 2014	0001
\$46,718.75	30 June 2014	0001

Year Three \$186,875.00

<u>Amount</u>	<u>Invoice Submission Date (on or about)</u>	<u>CLIN</u>
\$46,718.75	30 September 2014	0001
\$46,718.75	30 December 2014	0001
\$46,718.75	30 March 2015	0001
\$46,718.75	30 June 2015	0001

## ELECTRONIC INVOICING INSTRUCTIONS (JANUARY 2012)

**Wide Area Work Flow (WAWF) is the required method to electronically process recipient request for payment.** WAWF allows DOD recipients to submit and track invoices electronically.

Recipients shall (i) register to use WAWF at <https://wawf.eb.mil> and (ii) ensure an electronic business point of contact (POC) is designated in the System for Award Management site at <https://www.sam.gov> within ten (10) calendar days after award of this Assistance Agreement.

Questions concerning specific payments should be directed to the Defense Finance and Accounting Service (DFAS) Rome at 1-800-553-0527. You can also access payment and receipt information using the DFAS web site at <http://www.dfas.mil/dfas/contractorsvendors.html>. The award number or invoice number will be required to inquire about the status of your payment.

**The following codes and information are required to initiate the invoice and assure successful flow of WAWF documents.**

TYPE OF DOCUMENT: Grant and Cooperative Agreement Voucher

CAGE CODE: **0NJ12**

ISSUE BY DODAAC: **W81XWH**

ADMIN BY DODAAC: **W81XWH**

INSPECT BY DODAAC: **W81XWH**

ACCEPT BY DODAAC: **W81XWH**

SHIP TO DODAAC: **W81XWH**

LOCAL PROCESSING OFFICE DODDAC: **Not Applicable**

PAYMENT OFFICE FISCAL STATION CODE: **HQ0302**

EMAIL POINTS OF CONTACT LISTING:

INSPECTOR: [Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil](mailto:Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil)

ACCEPTOR: [Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil](mailto:Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil)

RECEIVING OFFICE POC: [Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil](mailto:Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil)

CONTRACT ADMINISTRATOR: **Leave Blank**

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ADDITIONAL CONTACT: [Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil](mailto:Usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil)

#### **AWARD CLOSE OUT**

a. The following documents shall be submitted within 90 calendar days of the end of the term of the award:

(1) Final SF425, "Federal Financial Report." Submit to:  
<https://www.usamraa.army.mil/pages/sf425>. Form and instructions are available on the web site.

(2) Final Technical Report. Submit to <https://ers.amedd.army.mil>.

(3) Final DD Form 882, "Report of Inventions and Subcontracts" (form available on web site <https://www.usamraa.army.mil>). Submit to [usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil](mailto:usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil).

(4) Cumulative listing of only the nonexpendable personal property acquired with award funds for which title has not been vested to the recipient, if applicable. This may be submitted on institution letterhead. Submit to [usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil](mailto:usarmy.detrick.medcom-usamraa.mbx.aa1@mail.mil).

b. In the event a final audit has not been performed prior to the closeout of the award, the sponsoring agency retains the right to recover an appropriate amount after fully considering the recommendations on disallowed costs resulting from the final audit.

c. The recipient shall promptly refund any unspent balances of funds the DOD Component has paid that is not authorized to be retained by the recipient. **Make check payable to the U.S. Treasury and mail to:**

USAMRAA  
Attn: MCMR-AAP-C  
Award No. W81XWH-12-1-0506  
820 Chandler Street  
Fort Detrick, Maryland 21702-5014

(End of Summary of Changes)

## Appendix C. Manuscript under review





## Vortioxetine Inhibits Marble-Burying and Transiently Enhances Social Sniffing in Adult Male BTBR Mice

Journal:	<i>Autism Research</i>
Manuscript ID	AUR-15-0263
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	15-Dec-2015
Complete List of Authors:	Lee, Benita; The University of Texas Health Science Center at San Antonio, Physiology Pehrson, Alan; Lundbeck Research USA Inc, External Sourcing & Scientific Excellence Witt, Nasriya; The University of Texas Health Science Center at San Antonio, Physiology; University of Texas at San Antonio, Biology Allen, Jonathan; The University of Texas Health Science Center at San Antonio, Physiology; University of Texas at San Antonio, Biology Sanchez, Connie; Lundbeck Research USA Inc, External Sourcing & Scientific Excellence Gould, Georgianna; The University of Texas Health Science Center at San Antonio, Physiology
Keywords:	Animal Models, Restricted/Repetitive Behaviors, Social Cognition, Serotonin < Neurochemistry, Lu AA21004, Pharmacology

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**Vortioxetine Inhibits Marble-Burying and Transiently Enhances Social Sniffing in Adult Male BTBR Mice**

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**Running Title:** Vortioxetine on BTBR compulsive & social behaviors

**Number of text pages:** 25

**Number of Tables:** 1

**Number of Figures:** 3

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Grant Sponsor: Max and Minnie Tomerlin Voelcker Fund. Grant Sponsor: Congressionally Directed Medical Research Program; Grant Number AR110109. Grant Sponsor: National Institute of Health; Grant Number R21HD081261.

**Scientific Summary Title:** New Multimodal Antidepressant Reduces Compulsive Behavior and Transiently Enhances Social Behaviors in a Mouse Model of Autism

**Lay Abstract:** Vortioxetine is a multimodal antidepressant that functions like Prozac (fluoxetine), because it blocks the serotonin transporter responsible for clearing serotonin released in the brain. By blocking serotonin clearance, excitatory neurotransmission is prolonged, which may promote improvements in mood and social behavior. Prozac is used to manage behavioral symptoms of autism effectively in some patients, but its broader beneficial effects are limited. Vortioxetine differs from Prozac because it also binds to several serotonin receptor subtypes, and it exerts radically different effects at them. For example, vortioxetine boosts the effects of serotonin at its receptor types 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> and this may stimulate social interest. At the same time, it suppresses serotonin's effects at 5-HT<sub>1D</sub>, 5-HT<sub>3</sub>, and 5-HT<sub>7</sub> receptors which may reduce anxiety, seizures, gastrointestinal disturbances, and most pertinently compulsive behaviors. Given these remarkable properties, we hypothesized that vortioxetine might alleviate both compulsive and social behavioral symptoms of autism, and we tested it in adult male BTBR mice. BTBR are an inbred model of impaired social behavior and compulsive behaviors of unknown etiology that is widely used in autism research. BTBR mice were dosed with vortioxetine acutely, and it significantly reduced their compulsive marble burying. It also enhanced social behavior, but this was a short lived effect. Brains were collected after behavior tests to measure vortioxetine's occupancy of its target binding sites, which was sufficient to correspond to the behavioral effects observed. Overall, these findings indicate vortioxetine has potential sociability-enhancing and restrictive-repetitive behavior suppressing properties that might comprehensively manage autism's core symptoms.

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**Scientific Abstract:** Vortioxetine is a multimodal antidepressant that inhibits the serotonin (5-HT) transporter (SERT), is an agonist at 5-HT<sub>1A</sub> receptors, a partial agonist at 5-HT<sub>1B</sub> receptors and is an antagonist at 5-HT<sub>1D</sub>, 5-HT<sub>3</sub> and 5-HT<sub>7</sub> receptors. In prior studies the selective serotonin reuptake inhibitor (SSRI) fluoxetine and the 5-HT<sub>1A</sub> receptor agonist buspirone improved sociability, but not a compulsive behavior (marble burying) in BTBR T<sup>+</sup>Itpr<sup>3</sup>tf/J (BTBR) mice. We hypothesized that vortioxetine, with its multimodal mechanism of action, might improve sociability and also reduce marble burying in adult male BTBR mice. Vortioxetine (5 or 10 mg/kg) was administered 30 or 60 min before three-chamber sociability tests, and 75 or 105 min before marble burying tests. In interaction preference tests, vortioxetine 10 mg/kg (but not 5 mg/kg) administered 30 min before testing enhanced social sniffing (p<0.05) relative to vehicle controls. This sociability enhancing effect was lost if vortioxetine was administered 60 min before testing. Vortioxetine (10 mg/kg) significantly reduced marble burying (p<0.02) at 75 and 105 min after administration, perhaps due to 5-HT<sub>7</sub> receptor antagonism. At 110 min after administration, 10 mg/kg vortioxetine had occupancy levels (%) of 84 ± 1, 31 ± 12 and 80 ± 5 of SERT, 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors in brain regions with high binding site densities. Overall, these findings indicate vortioxetine has potential sociability-enhancing and restrictive-repetitive behavior suppressing properties in BTBR mice. This warrants further investigation of vortioxetine as a potential treatment for core autism symptoms in BTBR and other mouse models under different conditions, and perhaps also in controlled clinical studies in adults.

**Key Words:** animal models, restricted/repetitive, social cognition, serotonin, multimodal antidepressant, Lu AA21004

## 1. Introduction

Social behavior deficits are the most treatment-resistant of autism core symptoms. There is much evidence that central serotonin (5-HT) system dysfunction, specifically insufficient 5-HT neurotransmission, contributes to the persistent sociability deficits of autism (Lucki 1998; Murphy et al., 2004; Santangelo and Tsatsanis 2005; Lam et al., 2006; Zafeiriou et al., 2009). The 5-HT transporter (SERT) is a major regulator of 5-HT transmission in the brain. SERT takes up extracellular 5-HT with high affinity back into 5-HT neurons to clear it from synapses and extracellular fluid. Clinical studies show autism symptoms worsen with decreases in 5-HT availability, but improve for some patients following administration of the selective 5-HT reuptake inhibitor (SSRI) fluoxetine (McDougle et al., 1996; West et al., 2009a&b; Hollander et al., 2012). However other SSRIs have had adverse outcomes, and as a class SSRIs by themselves have limited therapeutic effectiveness in in patients with autism (Henry et al., 2009; West et al., 2009b; Williams et al., 2013).

Restrictive-repetitive behaviors, the other core autism symptom, are less responsive to SSRI treatments than obsessive compulsive disorder (OCD) symptoms, which may relate to the finding that patients with autism suffer more from compulsions than obsessions (McDougle et al., 2000; 2005). Restrictive-repetitive compulsions in autism are most often controlled using antipsychotics such as risperidone, which acts as a potent 5-HT<sub>2</sub> receptor antagonist, dopamine D<sub>2</sub> antagonist as well as an antagonist at other non-serotonergic receptors (McDougle et al, 2005; Soorya et al., 2008; Kozielska et al., 2012). However, a recent study shows that antagonism or partial-agonism of other 5-HT receptor subtypes such as 5-HT<sub>7</sub> may also combat restrictive-repetitive tendencies (Canal et al., 2015).

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Vortioxetine is a multimodal antidepressant that combines SERT inhibition with 5-HT<sub>1A</sub> receptor agonism; it also acts as a 5-HT<sub>1D</sub>, 5-HT<sub>3</sub> and 5-HT<sub>7</sub> receptor antagonist, and as a partial agonist at 5-HT<sub>1B</sub> receptors (Sanchez et al., 2015). Acute and sub-chronic administration of vortioxetine to rodents has shown increased activity of several neurotransmitter systems, including a serotonergic enhancement beyond that of an SSRI in brain areas such as the prefrontal cortex (Bang-Andersen et al., 2011; Sanchez et al., 2015). Vortioxetine also produced antidepressant- and anxiolytic-like effects in several translational behavioral tests in rodents (Mørk et al., 2012; Guilloux et al., 2013).

widely used model in autism research because it exhibits impaired social interactions and restricted-repetitive behaviors that are often responsive to drug treatments (Moy et al., 2007; McFarlane et al., 2008; Meyza et al., 2013). The SSRI fluoxetine and the 5-HT<sub>1A</sub> receptor partial-agonist buspirone acutely improved social interaction preference in BTBR mice, as did tryptophan supplementation (Gould et al., 2011; Zhang et al., 2015), whereas repetitive behaviors, by measure of marble burying, were not responsive to the same serotonergic manipulations, but were reduced by risperidone treatment (Gould et al., 2011). Also, post-synaptic 5-HT<sub>1A</sub> receptors appear to be upregulated in the BTBR hippocampus, potentially conferring enhanced sensitivity to 5-HT in this region (Gould et al., 2014). Taken together, these findings support the idea that serotonergic manipulations of social and repetitive behaviors relevant to autism can be effectively studied in BTBR mice, to aid in developing improved therapies for some patient populations with autism.

Given vortioxetine's activity at SERT, 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors, and the prior findings with fluoxetine and buspirone in BTBR mice, we hypothesized that it would improve

sociability. Because of vortioxetine's modulation of other 5-HT receptor subtypes and neurotransmitter systems beyond the serotonergic, we also explored its capability to reduce repetitive marble burying in this strain.

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**2. Methods**

*2.1. Animals:* All procedures involving live mice were reviewed and approved by the University of Texas Health Science Center Institutional Animal Care and Use Committee. Subjects were 4-6 months old BTBR male mice bred in-house from colony founders purchased from Jackson Labs (Bar Harbor, ME). Stimulus ‘stranger’ mice for the sociability tests were 8-10 weeks-old male 129S1/SvImJ from Jackson Labs, kept in cages on a different ventilated housing rack than the subjects. Strangers were pre-conditioned for testing by 3 x 30 min confinement sessions under wire cup cages. Mice were maintained on a 14:10 h light-dark cycle, with lights on at 0700 h, *ad-libitum* access to chow (Teklad #7912, Harlan, Madison, WI) and fresh water, at room temperature (22-25°C). Mice were housed 3-5 per cage with male siblings in wood-chip bedding that was changed every week.

*2.2. Drug Treatments:* On the day of behavioral testing, the mice were brought to the procedure room 1 h before handling. The mice were weighed and injected in the abdominal interperitoneal (i.p.) area with either 5 or 10 mg/kg vortioxetine (Lundbeck, Copenhagen, Denmark) that was first dissolved in 100% DMSO and subsequently diluted to 5% with saline, or with 5% DMSO in saline for vehicle controls. Vortioxetine-treated mice weighed 34±1 g on average and vehicle control average weight was 37±1 g. Tails were marked with colored permanent ink for identification and subjects were returned to home cages for either 30 or 60 min.

*2.3. Three-Chamber Sociability Tests:* Social interaction and social novelty preference tests were performed between 1200 and 1600 h, starting 30 or 60 min post-injection, as described in previous studies (Gould et al., 2011; 2014). The acrylic testing arenas (60 x 30 x 22 cm) had black outer sides, transparent interior walls with doors, and tan floors. Tests were performed



under low intensity red light (16 lux). Subjects acclimated in the center for 10 min, after which the doors to the outer chambers were opened so subjects could explore the entire empty test arena for 10 min. To test social interaction preference, the subject was confined to the center while an empty wire cage (novel object) and a stranger (stranger #1) in a wire cage were placed in opposite end chambers. Stimuli placement was randomized and balanced between groups. Subjects were then released to explore the arena for 10 min and their behavior was video recorded. Preference for social novelty was tested by confining subjects in the center chamber, while a new stranger (stranger #2) was placed in empty cages and the “old” stranger (stranger #1 from interaction preference test) was re-positioned in the chamber. Subjects were released again and their preference for old *versus* new strangers was video recorded for 10 min. Strangers were returned to their home cages while arenas and cup-cages were cleaned between tests of subjects. Observers blinded to the treatment collected data from the video recordings, which included time spent in each chamber, number of chamber entries, and time engaged in sniffing of stranger mice or objects.

**2.4. Marble Burying:** Immediately after the sociability tests (75 or 105 min post-injection), subjects were transferred to clean 50 x 28 x 23 cm transparent cages filled to a depth of 10 cm with wood chip bedding. Fifteen flattened blue glass marbles were spaced evenly on the bedding in a 3 x 5 grid. Cages were covered with filter-tops and mice were permitted 30 min to bury marbles, as in Gould et al. (2014).

**2.5. Target Occupancy Measures:** Following the marble burying test (105 or 135 min post-injection, mice were euthanized by cervical dislocation. Immediately thereafter, brains were removed, flash frozen on powdered dry ice, and stored at -80°C in scintillation vials. Target occupancy experiments were performed using *ex vivo* autoradiography for mice treated with

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vortioxetine 10 mg/kg. Brains were cut in 20  $\mu$ m thick coronal slices, mounted onto slides and frozen at -20°C until used in the autoradiography experiments. [<sup>3</sup>H] escitalopram was used to assess SERT occupancy, while [<sup>3</sup>H]GR125743 was used to determine 5-HT<sub>1B</sub> receptor occupancy and [<sup>3</sup>H] WAY-100635 was used for 5-HT<sub>1A</sub> receptor occupancy, as described in detail in prior studies (Pehrson et al., 2013, Guilloux et al., 2013; Wallace et al., 2014).

*2.6. Statistical Analysis:* Repeated measures analysis of variance (RM-MANOVA) was used to compare social preference for chamber dwelling and sniffing, and ANOVA to compare marble burying and chamber entries. Significant differences were assessed either by t-tests or Fisher’s least significant difference (LSD) using Statistica (Statsoft, Tulsa, OK, USA).

### 3. Results

**3.1. Sociability Tests, 30 Min Post-Injection Delay:** In the three-chamber sociability tests, global observation of mice treated with 5 or 10 mg/kg vortioxetine or vehicle for time engaged in social sniff ( $F(1,34)=52$ ,  $p<0.001$ ) or spent in each chamber ( $F(1,34)=9.8$ ,  $p<0.003$ ) revealed significant interactions between test-phase and chamber preferences. The interactions aligned such that most mice, irrespective of treatment, preferred to investigate strangers *versus* novel objects in the first or social interaction phase, and this preference shifted in the second, social novelty phase toward “new” strangers *versus* the “old” strangers from the prior test (Fig. 1). The behavior of 15 vehicle controls, 12 mice treated with 5 mg/kg vortioxetine, and 10 mice treated with 10 mg/kg vortioxetine was included in the analysis. One vehicle control was excluded from the analysis for making only chamber entries, and two others were excluded because one subject jumped out of its test arena and landed in the arena of the other subject.

In the social interaction test, mice treated with 10 mg/kg vortioxetine exhibited significant preference for social interaction in their sniffing relative to vehicle controls (Fig 1a.  $F(1,34)=12$ ,  $p<0.001$ ). This was due to their spending less time sniffing empty cages ( $F(2,34)=3.3$ ,  $p\leq 0.05$ , LSD  $p<0.02$ ), since the time spent sniffing stranger mice was similar across groups. No treatment group displayed a social interaction preference by measure of time spent in chambers. However, mice treated with 10 mg/kg vortioxetine exhibited a non-significant trend toward interaction preference (Fig.1b,  $F(1,34)=3.5$ ,  $p=0.07$ ;  $t(9)=1.5$ ,  $p=0.1$ ). Mice treated with either 5 or 10 mg/kg vortioxetine made significantly fewer chamber entries during interaction tests ( $39\pm 5$  or  $32\pm 4$  entries) as compared to vehicle controls ( $53\pm 6$  entries,  $F(2,34)=4.4$ ,  $p<0.05$ ).

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In social novelty preference tests, all mice exhibited a novelty preference in their social sniffing, irrespective of treatment ( $F(1,34)=27$ ,  $p<0.001$ ,  $t(9-14)=2.3-4.6$ ,  $p<0.05$ , Fig. 1c). However, there was a significant interaction between treatment groups for time spent in chambers, such that only vehicle controls displayed a preference for social novelty ( $F(2,34)=3.5$ ,  $p<0.03$ ,  $t(14)=4.3$ ,  $p<0.001$ ), as shown in Fig. 1d. In this phase of the test, there was no difference between groups in the number of chamber entries, which had a mean of  $48\pm3$  entries for all groups.

*3.2. Sociability Tests With 10 mg/kg Vortioxetine, 60 Min Post-Injection Delay:* To further explore the temporal sensitivity of vortioxetine's impact on sociability, a second group of 12 mice treated with 10 mg/kg vortioxetine and 12 vehicle controls were compared for sociability 60 min post-injection. Data are shown in Fig. 2. Global analysis of sociability tests revealed significant interactions between test-phase and sniffing preference ( $F(1,22)=19$ ,  $p<0.001$ ) consistent with a shift in interest toward a novel stranger in the second phase of sociability testing. There was also a significant interaction between test phase, treatment and time in chambers ( $F(1,22)=5.3$ ,  $p<0.05$ ).

Sniffing time in the 60 min post-injection social interaction test was less than half that of the 30 min post-treatment experiment. Vortioxetine (10 mg/kg) significantly reduced social sniffing time relative to controls ( $F(1,22)=5.15$ ,  $p<0.05$ ; Fig. 2a), but had no impact on time spent sniffing novel objects. Time in chambers with novel objects was significantly increased by the 60 min wait following 10 mg/kg vortioxetine administration ( $F(1,22)=9.8$ ,  $p<0.05$ ), and time spent in chambers with strangers was also significantly reduced ( $F(1,22)=10.3$ ,  $p<0.05$ ) relative to vehicle controls (Fig. 2b). The number of chamber entries were similar across

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3 treatment groups ( $F(1,22)=0.8$ ,  $p=0.38$ ) with  $68\pm7$  entries for vehicle controls and  $61\pm5$  entries  
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5 for vortioxetine-treated mice.  
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8 Both 60 min post-injection groups (5 and 10 mg/kg vortioxetine) exhibited a preference  
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10 for social novelty in sniffing ( $F(1,22)=32$ ,  $p<0.001$ ;  $t(11)=6$ ,  $p<0.01$  for both doses, Fig. 2c) and  
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12 in chamber dwelling ( $F(1,22)=13$ ,  $p<0.01$ ;  $t(11)=2.4$ ,  $p<0.05$ , Fig. 2d). The number of chamber  
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14 entries made by vortioxetine ( $64\pm5$ ) or vehicle control mice ( $59\pm5$ ) during the tests did not  
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16 significantly differ ( $p=0.5$ ).  
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21 3.3. *Marble Burying*: Vortioxetine treated mice at 10 mg/kg, but not 5 mg/kg, buried  
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23 significantly fewer marbles than vehicle controls 80 min after injection ( $F(2,34)=3.4$ ,  $p<0.05$ )  
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25 and 110 min after injection ( $F(1,22)=10$ ,  $p<0.01$ ), as shown in Fig. 3.  
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29 3.4 *Target Occupancy by Vortioxetine*: SERT, 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptor occupancy levels  
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31 produced by vortioxetine 10 mg/kg at 110 and 140 min after an i.p. injection are shown in  
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33 Table 1. These data indicate that this dose of vortioxetine 10 mg/kg is associated with  $\geq 80\%$   
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35 occupancy of SERT and 5-HT<sub>1B</sub> receptors, and  $\sim 30\%$  occupancy of 5-HT<sub>1A</sub> receptors.  
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**4. Discussion**

*4.1. Social interaction and novelty preferences:* In this study BTBR mice administered vortioxetine at 10 mg/kg, but not 5 mg/kg, significantly favored social sniffing over investigation of a novel object when testing began 30 min post injection (fig 1a). However, when arena acclimation and sociability testing 60 min following i.p. administration of 10 mg/kg vortioxetine, we observed a different pattern of effects: Specifically, the preference for social interaction observed at 10 mg/kg, 30 min post injection was no longer present, and furthermore social sniffing was significantly reduced in the vortioxetine treatment group. In the 30 min post-injection start-time group, chamber entries were significantly lower than controls, whereas in the 60 min post-injection start-time group they were not. A 30 min wait in the home cage prior to arena conditioning is commonly used with drug treatments in three chamber sociability tests (Gould et al., 2011; Silverman et al. 2010). Earlier findings with fluoxetine and buspirone in BTBR mice were not tested with 60 min post-injection wait, so it is not clear if their sociability enhancement effects are similarly transient. This finding indicates it is critical to report and carefully control the time course of sociability testing in similar studies, since it may influence the interpretation of drug effects.

Alternatively, given the complex pharmacological profile of vortioxetine, the relative contribution of vortioxetine’s receptor mechanisms could differ at these two time points to produce opposite directed net effects unique to this drug. Time course studies of target engagement and brain exposure in rodents have shown that vortioxetine does not reach maximum levels until approximately 2-4 hours after a subcutaneous administration (Pehrson, unpublished observation). Since the 30 and 60 min time points are on the upward phase of the target engagement/exposure gradient, it is likely that the relative target occupancies could

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3 differ at critical times during sociability testing for these mice. Given the potentially promising  
4 effects with vortioxetine early on, and their radical loss with a 30 min delay in this pilot study,  
5 more extensive sociability tests including a wider range of post-injection start times and  
6 vortioxetine doses are warranted to elucidate these relations. Furthermore, before vortioxetine  
7 is tested to manage the core symptoms of autism in clinical trial, chronic treatment effects  
8 should also be explored pre-clinically as they may differ from acute effects.  
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18 Rodent studies demonstrate that stimulating 5-HT<sub>1A</sub> receptors can enhance sociability,  
19 particularly in models with other signs of relatively low 5-HT neurotransmission (Gould et al.,  
20 2011; Wang et al 2013). It may also promote social behavior beneficially in other contexts, for  
21 example buspirone ameliorated isolation-induced aggression with an ED<sub>50</sub> of 2.5 mg/kg in  
22 mice (Sanchez et al., 1993). While the enhancement of social interaction by vortioxetine (10  
23 mg/kg) was not as robust as with fluoxetine (10 mg/kg) or buspirone (2 mg/kg) administration  
24 in BTBR mice (Gould et al., 2011), it should be kept in mind that the drug-specific nature of 5-  
25 HT<sub>1A</sub> receptor occupancy may play a critical role in shaping social behavior. For example,  
26 buspirone is a partial agonist at 5-HT<sub>1A</sub> receptors, and while it enhanced BTBR sociability at 2  
27 mg/kg (tests began 30 min post-injection, Gould et al., 2011), in other studies it inhibited or did  
28 not affect social behavior at doses at or above 2.5 mg/kg (File and Seth, 2003). This  
29 observation could be driven by a number of mechanisms, including: 1) differing effects of  
30 moderate vs. high levels of 5-HT<sub>1A</sub> receptor occupancy, 2) engagement of differing  
31 subpopulations of 5-HT<sub>1A</sub> receptors, e.g. postsynaptic ones tied to inwardly rectifying K<sup>+</sup>  
32 channels, versus autoreceptors tied to Gi/o proteins, or 3) the engagement of other lower  
33 affinity targets of buspirone with opposing network-level actions.  
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By contrast, vortioxetine is a full agonist at 5-HT<sub>1A</sub> receptors, and the contribution of this mechanism necessarily impacts other serotonergic effects on BTBR sociability. Our dose selection was based on the finding that 2 mg/kg of vortioxetine promoted social interactions in rat open-field tests (Mørk et al., 2012). However, these conditions may not translate into optimal sociability-enhancing doses for the BTBR mouse model, in which 5-HT<sub>1A</sub> receptors could have a different affinity for vortioxetine due to elevated corticosterone levels or other gene or physiologically-based differences in receptor function as compared to other mouse strains (Gould et al., 2014). Furthermore, drug affinities for rodent and human 5-HT<sub>1A</sub> receptors differ (Newman-Tancredi et al., 2005). Indeed, vortioxetine's affinity for 5-HT<sub>1A</sub> receptors in rat and mouse brain tissue is 10 to 15-fold lower than for the human receptor (Sanchez et al., 2015). Thus, our findings in mice likely underestimate the contribution that this receptor mechanism might have in human therapeutics. One way to address this in future studies would be to dose with a combination of vortioxetine and a 5-HT<sub>1A</sub> receptor agonist to mimic human levels of 5-HT<sub>1A</sub> receptor occupancy in the mouse.

Another potential explanation for vortioxetine's short-lived beneficial effects in social preference could be related to acetylcholinergic neurotransmission. Previous studies have demonstrated that BTBR mice have lower extracellular basal acetylcholine levels in the medial prefrontal cortex than more sociable C57BL/6 mice (Mc Tighe et al., 2013). Vortioxetine treatment within the dose range used in the present study produces a small yet significant increase in extracellular acetylcholine in the rat medial prefrontal cortex, which also shared a similarly brief timecourse of effect (Mork et al., 2013). Taken together with the observation that acholinesterase inhibition by repeated administration of donepezil also enhances sociability in BTBR mice (Karvat and Kimchi, 2014), it becomes plausible that vortioxetine's transient



sociability enhancing effects may be mediated by cholinergic mechanisms. The relevance and plausibility of cholinergic interventions for the symptoms of autism is thus in need of further study.

*4.2. Social novelty preference:* At 30 min post-injection, both vortioxetine doses produced a partial loss of social novelty preference. While mice treated with vehicle, 5 or 10 mg/kg vortioxetine all spent significantly more time sniffing a novel stranger by comparison to a stranger previously encountered in the interaction test, the vortioxetine-treated mice spent the same amount of time in new and old stranger chambers while by contrast vehicle treated mice spent more time with the novel stranger mouse (Fig 1d). Previously we observed that fluoxetine and buspirone reduced social novelty preference by measure of time in chambers, and reduced social sniffing in novelty preference tests (Gould et al., 2011). Interestingly, in the present study BTBR mice given 10 mg/kg vortioxetine and a 60 min post-injection start time exhibited no loss of social novelty preference (Fig 2c&d), in contrast to the 30 min post-injection start-time groups. A prolonged lag following handling and i.p. injection is also likely to be associated with higher plasma corticosterone levels that may influence social exploration during the novelty phase of testing (Gould et al., 2014). This conjecture also remains to be tested empirically.

*4.3 Marble Burying:* Effects of vortioxetine in this study on BTBR marble burying were less time-sensitive, 10 mg/kg treatments at either time reduced the behavior significantly. The mouse marble burying test is based on a natural investigative behavior that turns compulsive in some strains. It is an index of repetitive or compulsive behavior that is divorced from anxiety and is generally suppressed by antipsychotics and anxiolytics, synthetic cannabinoid agonists,

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acetaminophen and some SSRIs (Deacon, 2006; Thomas et al., 2009; Gould et al., 2012; Albelda and Joel 2012). The SSRI fluoxetine and partial 5-HT<sub>1A</sub> agonist buspirone both failed reduce marble burying by BTBR mice when given acutely at single doses enhancing their preference for social interaction (Gould et al., 2011). By contrast, risperidone reduced marble-burying at a dose of 0.1 mg/kg, whereas it suppressed locomotor behavior at  $\geq 1$  mg/kg (Silverman et al., 2010; Gould et al. 2011). Although risperidone has high affinity for 5-HT<sub>1A</sub> receptors, it does not act as a 5-HT<sub>1A</sub> agonist or partial agonist (Newman-Tancredi et al., other studies show 5-HT<sub>1A</sub> agonists and partial agonists inhibit marble burying and other obsessive-compulsive index behaviors in mice, while 5-HT<sub>1B/1D</sub> and 5-HT<sub>3</sub> targeting generally does not affect them (Gaikwad et al., 2010, Tsaltas et al., 2005). A study using knock-out mice and antagonists demonstrates that 5-HT<sub>7</sub> receptors regulate mouse marble-burying (Helund and Sutcliffe, 2007). Indeed, targeting 5-HT<sub>7</sub> and 5-HT<sub>1A</sub> receptors by the novel partial agonist +5-(2'-fluorophenyl)-2-dimethylaminotetralin (+)-5-FTP corrected innate or drug-induced motor stereotypies in C58/J and C57BL/6J mice (Canal et al., 2015). Targeting of these receptor subtypes in opposing ways by vortioxetine may contribute to or underlie its very promising dose-dependent inhibition of BTBR marble burying.

**Conclusions:** In sum, vortioxetine appears to hold some promise for the treatment of sociability impairments and/or repetitive behaviors in autism spectrum disorders. Further pre-clinical studies over a longer time course, a broader dose range and chronic dosing will be required to fully assess the impact of vortioxetine on sociability. Conservatively, autism spectrum disorders affect 1.3-6 per 1,000, or roughly 1 in 110 children under the age of 6 years in the United States (Fombonne, 2005; CDC, 2009). Of these, roughly 20-40% of

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3 individuals with autism are likely to benefit from treatments that boost the capacity for 5-HT  
4 neurotransmission (Veenstra-VanderWeele and Blakely, 2012; Hammock et al., 2012; Kerr et  
5 al., 2013). Based on our findings with vortioxetine, it and possibly other multimodal serotonin-  
6 based antidepressants maybe of some benefit to patient sub-populations that exhibit  
7 symptoms consistent with low central serotonergic neurotransmission to ameliorate core  
8 autism symptoms.  
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## Figures:

### **Fig. 1. Impact of vortioxetine treatment 30 min prior to testing on BTBR sociability preferences.**

All data shown are means  $\pm$  standard error. (a) Vortioxetine at 10 mg/kg significantly enhanced social interaction preference as shown by social sniffing relative to vehicle treated controls. \*\*Significant preference for stranger versus novel object ( $p < 0.01$ ). (b) Measures of time spent in chambers revealed no social interaction preferences; however, vortioxetine-treated mice made 25-40% fewer chamber entries during this phase of testing. (c) Vortioxetine and vehicle-treated mice exhibited similar preferences for social novelty by social sniffing measures in which all groups spent significantly ( $*p < 0.05$ ) more time sniffing newly introduced versus “old” strangers from the prior interaction tests. (d) Only vehicle-treated mice spent significantly ( $*p < 0.05$ ) more time in chambers with new versus old strangers. By the second phase of sociability testing, chamber entries made by all treatment groups were similar.

### **Fig. 2. Impact of 10 mg/kg vortioxetine treatment 60 min prior to testing on BTBR sociability preferences.**

(a) Vortioxetine significantly reduced social sniffing and (b) dwelling near strangers relative to controls (\*\* significantly lower than control,  $p < 0.05$ ), and increased time spent in chambers with novel objects ( $*p < 0.05$ ) during the social interaction phase. (c) Both treatment groups had significant preferences for social novelty in sniffing and in (d) chamber dwelling time ( $*p < 0.05$ ). Chamber entries ranged from 59-68 and did not differ significantly between treatment groups and test phases.

**Fig. 3. Impact of vortioxetine treatment on marble burying behavior.** Vortioxetine (10 mg/kg) significantly reduced marble burying by BTBR relative to vehicle treated controls ( $*p < 0.05$ ). The same vehicle control group was used to compare the 5 and 10 mg/kg vortioxetine treatments at 80 min, a 5 mg/kg group was not tested at 110 min.

**Table 1. Occupancy of BTBR Serotonin Transporter and 5-HT Receptors by Vortioxetine.**

Serotonin transporter (SERT), 5-HT<sub>1B</sub> and 5-HT<sub>1A</sub> receptor occupancy by systemically administered vortioxetine was determined using quantitative autoradiography using saturating concentrations of labeled ligands selective for these targets. Reductions in radioligand binding from the vehicle mean due to competitive displacement by vortioxetine are expressed as % target occupancy. These did not differ significantly among the two times examined.

Treatment group	Target Occupancy (%) (mean ± SEM)		
	SERT	5-HT <sub>1B</sub> receptor	5-HT <sub>1A</sub> receptor
Vehicle	0 ± 2.3	0 ± 2.6	0 ± 15
Vortioxetine 10 mg/kg, 110 min post-i.p.	84 ± 1.1	80 ± 4.9	31 ± 12
10 mg/kg, 140 min post-i.p.	80 ± 2.7	78 ± 8	31 ± 8

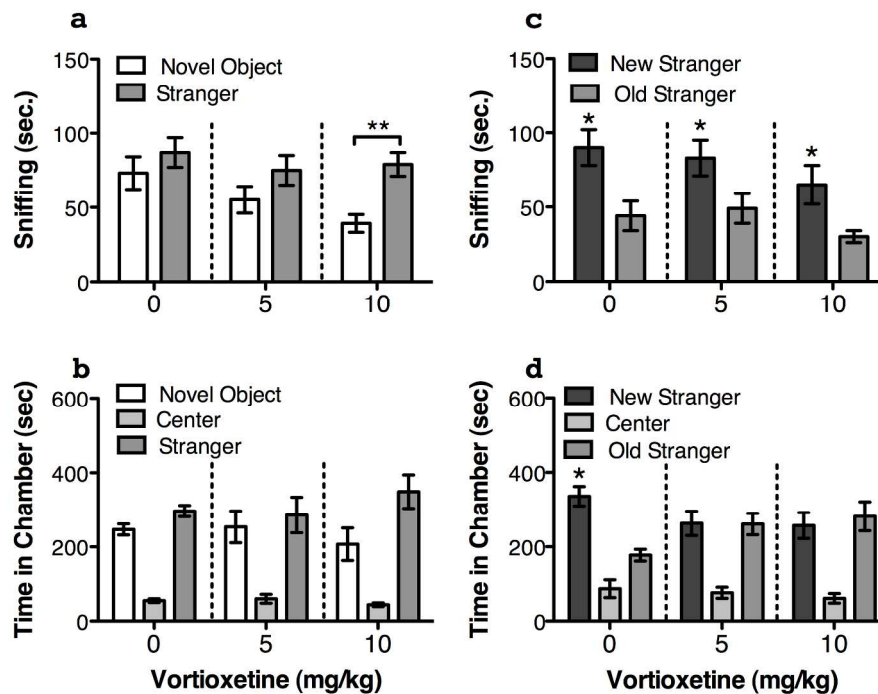


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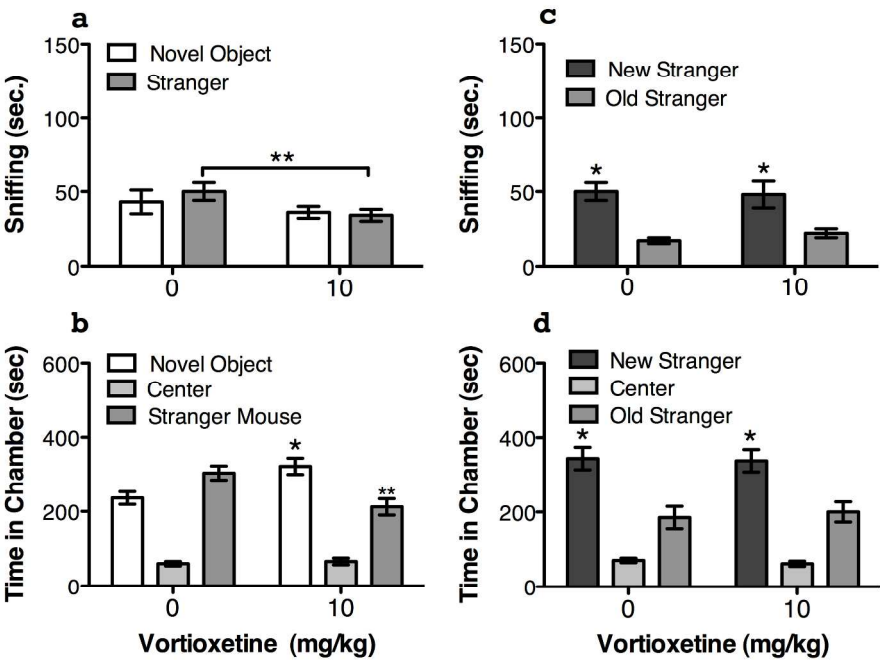


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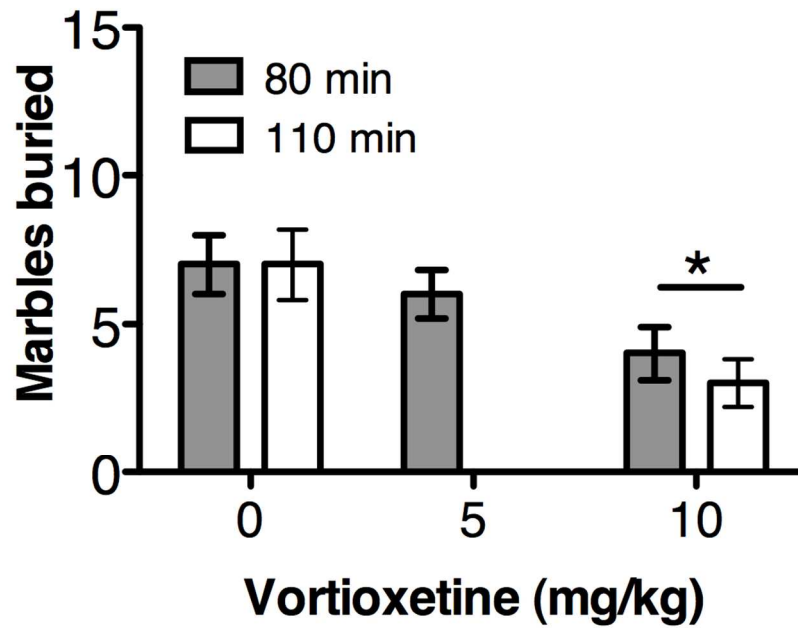


Fig. 3. Impact of vortioxetine treatment on marble burying behavior. Vortioxetine (10 mg/kg) significantly reduced marble burying by BTBR relative to vehicle treated controls (\*  $p < 0.05$ ). The same vehicle control group was used to compare the 5 and 10 mg/kg vortioxetine treatments at 80 min, a 5 mg/kg group was not tested at 110 min.

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## Appendix D. Poster presentation of berberine results

# Uptake 2 Blockade by Berberine Enhances Sociability and Reduces Dominance in Mice

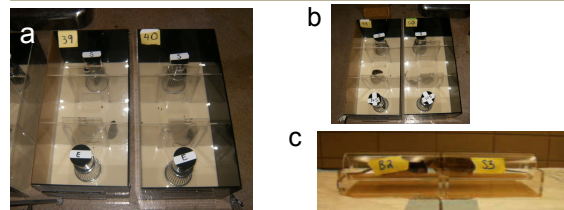
Alicia Sanchez<sup>1,2</sup>, Corey M. Smolik<sup>1</sup>, Timothy Pham<sup>1,3</sup>, Kiran Lalani<sup>1,3</sup>, Georgianna G. Gould<sup>1</sup>

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## Introduction

Autism is a developmental disorder classified by three core symptoms: impaired social interaction, impaired communication, and repetitive behaviors. We found the pseudoisocyanine decynium-22 (D-22) enhances social interactions in BTBR mice via 'uptake 2' blockade in a manner similar to fluoxetine, an antidepressant that blocks serotonin (5-HT) reuptake from extracellular fluid by the 5-HT transporter [1]. Uptake 2 in the brain includes organic cation transporters (OCTs) that remove monoamines from extracellular fluid with lower affinity but greater capacity than SERT. In this study, we sought to validate uptake 2 transporter blockade as a therapeutic mechanism to ameliorate social interaction deficits. Systemic administration of the alkaloid antimicrobial berberine produced antidepressant-like effects, and increased monoamine levels in mouse hippocampus and frontal cortex [2]. Berberine appears to be a blocker and substrate of murine OCT subtypes 2 and 3 [3,4]. We hypothesized that since it shared this mechanism of action with D-22, berberine might likewise improve social behavior in socially-deficient BTBR mice. We also examined berberine's effects on the social behaviors of typically gregarious C57BL/6 mice. Finally, we compared the baseline social behaviors of C57BL/6 mice from different suppliers (Jackson vs. Harlan) to assess the latter for use in future studies.

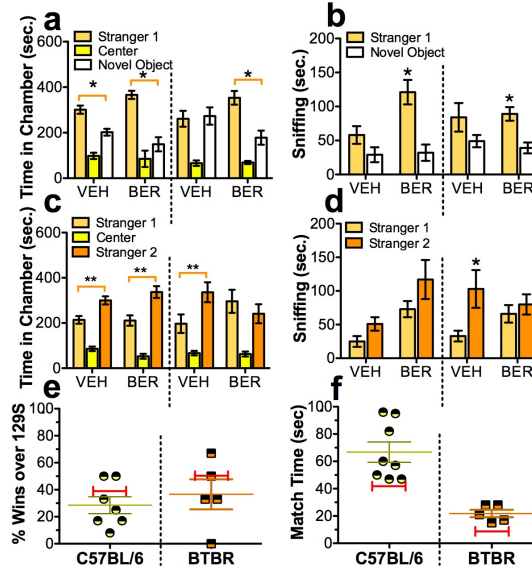
## Materials and Methods



**Fig. 1. Arenas for (a) social interaction or (b) novelty preference tests, and setup for the (c) tube test for social dominance.**

**Behavior:** Three-month old male BTBR or C57BL/6 mice were injected i.p. with 0.5 mg/kg berberine or vehicle (10DMSO in 90% saline) and returned to their home cage for 30 min. Then they were introduced into three-chamber arenas for 20 min to acclimate. Interaction preference tests commenced by introducing a stranger mouse under a cup cage at one end and an empty cage in the opposite arena end (Figure 1a), subjects freely explored while video-recorded for 10 min. Subsequently novelty preference tests were conducted by introducing a second stranger in the empty cage (Figure 1b) so the subject could investigate both strangers for 10 min. Social sniffing and time in chambers was recorded from videos and analyzed by repeated-measures ANOVA followed by Fisher's LSD or t-tests to compare means. Social dominance tube tests were performed as in [5], each subject was matched vs. 6 strangers to obtain scores based on advance or retreat (1 = win, 0 = loss, 0.5 = 3 min draw), outcomes vs. controls were assessed by  $\chi^2$  tests. Strangers in tests were age-matched 129S1/SvImJ males. **Synaptosomal [<sup>3</sup>H] 5-HT Uptake:** Capacity of berberine to block 5-HT uptake relative to D-22 was assessed in frontal cortex by 5 min competition assays conducted as described in [4].

## Results

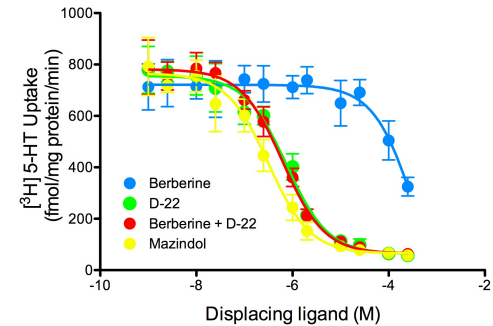
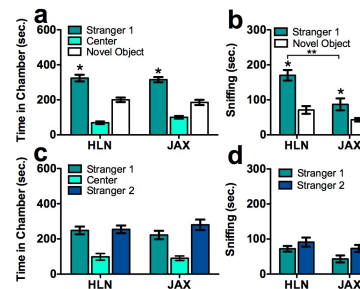


**Fig 2. Effects of berberine on social behaviors of adult male mice.**

(a) Berberine (0.5 mg/kg) enhanced the otherwise low BTBR preference for social interaction, but was without effect on this measure in C57BL/6 (\* $p < 0.05$  N=6-10). (b) Social sniffing was promoted by berberine in both strains. (c) BTBR mice lost inherent preference for social novelty with berberine treatment, while C57BL/6 mice did not. (d) Only BTBR mice exhibited strong preference for novelty in sniff tests. (e) Berberine made tube test losses more common in both strains relative to vehicle control (red = means) and (f) increased match duration ( $\chi^2 p < 0.01$ , N=5-7).

### Fig 3. C57BL/6 mice from different suppliers exhibit similar social behaviors.

Mice from Harlan (HLN) and Jackson (JAX) displayed similar preferences (\* $p < 0.05$ ) for (a) social interaction and (c,d) lack of novelty preferences. However, (b) HLN mice spent significantly (\* $p < 0.05$ ) more time sniffing novel "stranger 1" mice in the interaction test phase (N=8).



**Fig 4. Berberine is a less potent serotonin uptake blocker than D-22.**

In synaptosome preparations from BTBR mouse frontal cortex, the 'uptake 2' transporter blocker Decynium 22 (D-22) blocked serotonin (5-HT) uptake with a  $K_m \approx 0.5 \mu M$  that was comparable to the general 'uptake 1' monoamine reuptake inhibitor mazindol ( $K_m \approx 0.2 \mu M$ ). In contrast, berberine blocked 5-HT uptake with a  $K_m \approx 240 \mu M$  (N=3). Prior studies have shown that berberine is a substrate of OCTs [3] and that it may block norepinephrine uptake more potently than 5-HT [2].

## Conclusions

○Berberine and D-22 both inhibit OCT function and enhance sociability in socially-impaired BTBR mice.

○This supports the ideal that OCT blockade may be an effective pharmacological strategy to ameliorate prominent social behavior deficits that occur in autism or schizophrenia.

○D-22 is a better 5-HT uptake blocker than berberine.

○This raises the possibility that catecholamine uptake blockade may be an important mechanism contributing to the beneficial behavioral effects of OCT blockade.

○C57BL/6 mice from different suppliers exhibit similar social behaviors.

○This reduces prior concerns about genetic drift in this strain confounding behavior experiment outcomes.

## References and Acknowledgements

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- We thank Dr. Lyn Daws for suggestions and use of her laboratory facilities and Harlan for providing sample mice. This research was supported by 5R25NS080684-04 (START-UP Program, UTHSCSA) and W81XWH-12-1-0506 (CDMRP DOD Autism Idea Award).

## **Appendix E: Accepted Proposal for Symposium at “Serotonin in Seattle” July 24-27, 2016**

1. Symposium Title: "The Highs and Lows of Serotonin in Autism Spectrum Disorders"
2. Serotonin system disruptions are evident in a subset of individuals with autism spectrum disorders (ASD). Platelet hyperserotonemia was the first reported biomarker of autism, it manifests in about 30% of ASD patients. Neuroimaging and behavioral pharmacology studies also implicate serotonin system dysfunctions in ASD, although these findings do not fit neatly into a simple model. For example decreased serotonin transporter and receptor binding, altered synthetic capacity across development, and sensitivity to tryptophan depletion were reported in different human studies. This symposium will delve into genetic and physiological mechanisms by which the serotonin system can contribute to ASD risk. Mutations in mouse models that either promote or impair serotonin transporter (SERT) function alter social or repetitive behaviors. Dr. Veenstra-VanderWeele will discuss how neurodevelopment and behavior are affected by enhanced SERT capacity. Importantly, SERT function is influenced by integrins that regulate cell matrix interactions and influence synaptic structure and activity. Dr. Carneiro will present her findings on how the integrin  $\beta 3$  subunit modulates SERT activity. Also, mutations interfering with serotonin synthesis or metabolism can produce autism-like behaviors in mice. Dr. Dougherty will share his discoveries about the Celf6 mutation that triggers resistance to change, reduced vocalizations and low brain serotonin levels. Finally, either excessive or insufficient developmental serotonin availability can be key environmental contributors to autism-like behaviors. Dr. Bonnin will discuss how alterations in prenatal serotonin triggered by maternal stress, exposure to SERT blockers and other prenatal insults impact fetal brain development in mice and contribute to the onset of neurodevelopmental disorders.
3. Symposium Chair: Georgianna G. Gould, Ph.D.  
Position and Job Title: Research Associate Professor  
Affiliation: The University of Texas Health Science Center at San Antonio, Department of Physiology  
e-mail: [gouldg@uthscsa.edu](mailto:gouldg@uthscsa.edu)

Symposium Co-Chair: Lynette C. Daws, Ph.D.

Position and Job Title: Professor

Affiliation: The University of Texas Health Science Center at San Antonio, Department of Physiology

e-mail: [daws@uthscsa.edu](mailto:daws@uthscsa.edu)

### 4. Speakers:

Jeremy Veenstra-VanderWeele, MD

Talk title: Impact of increased SERT function on autism-related brain development and behavior.

Position and Job Title: Associate Professor and Research Psychiatrist

Affiliation: Columbia University and New York State Psychiatric Institute

e-mail: [veenstr@nyspi.columbia.edu](mailto:veenstr@nyspi.columbia.edu)

Ana Carneiro, Ph.D.

Talk title: Link between integrin  $\beta 3$ , hyperserotonemia and autism.

Position and Job title: Assistant Professor

Affiliation: Vanderbilt University, Department of Pharmacology

e-mail: [ana.carneiro@vanderbilt.edu](mailto:ana.carneiro@vanderbilt.edu)

Joseph Dougherty, Ph.D.

Talk title: Celf6 and prenatal SSRI manipulations

Position and Job Title: Assistant Professor

Affiliation: Washington University in St. Louis, Department of Genetics

e-mail: [jdougherty@genetics.wustl.edu](mailto:jdougherty@genetics.wustl.edu)

Alexandre Bonnin, Ph.D.

Talk title: Prenatal serotonin role in the fetal programming of mental disorders

Position and Job Title: Assistant Professor

Affiliation: Keck School of Medicine of University of Southern California

e-mail: [bonnin@med.usc.edu](mailto:bonnin@med.usc.edu)